

2008

Reductive Dehalogenation of Chlorinated Alkanes by Novel Bacteria at the PetroProcessor of Louisiana Inc. Superfund Site

Jun Yan

Louisiana State University and Agricultural and Mechanical College

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**REDUCTIVE DEHALOGENATION OF CHLORINATED ALKANES BY
NOVEL BACTERIA AT THE PETROPROCESSOR OF LOUISIANA INC.
SUPERFUND SITE**

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Civil and Environmental Engineering

by

Jun Yan

B.S., Nanjing University, China, 2000

M.S., Nanjing University, China, 2003

May 2009

ACKNOWLEDGMENTS

I would like to take this opportunity to express my sincere appreciation to my advisor, Dr. William M. Moe, for offering me such a great opportunity to complete my Ph.D. research under his direction. During my study at LSU, I have enjoyed my research on this project in Dr. Moe's group. His insightful guidance and generous support always inspire my research and encourage me to pursue the accomplishments of my career.

I would like to acknowledge Dr. Fred A. Rainey, for his instruction in the field of microbiology, molecular biology, and phylogenetic analysis in this research, and Dr. John H. Pardue for his instructions on GC-MS analysis of dehalogenation products. My appreciation is also given to Dr. W. David Constant and Dr. Mark Benfield for kindly serving on my dissertation committee.

I would like to thank the former and current members in our research group, Dr. Congna Li, Dr. Bing Qi, Dr. Hee-Sung Bae, Dr. Brian Rash, Kimberly Bowman, Dr. Jyoti U Rao, and Marilou Nabatilan for their supports and friendship. I would also like to thank the members from Dr. Pardue's group, Dr. Xingmao Ma, Dr. Han-Woong Lee, Stephen E. Mbuligwe and Dr. Eun-Ju Lee, and the members of Raineylab, Dr. Manish Shukala and Ryan Callegan.

My appreciation also extends to Cindy Henk from the LSU Socolofsky Microscopy Center, and Dr. William G. Henk from the LSU School of Veterinary Medicine for assistance in preparing cell samples and taking microscopy (SEM and TEM) images of bacteria isolated in this research. Special thanks are given to Dr. Milton S. da Costa and his group from Universidade de Coimbra, Portugal, for analysis of DNA G+C content; Dr. Kenneth E. Damann, Jr. from the LSU Department of Plant Pathology and Crop Physiology, and Dr. Gregg S. Pettis and his graduate student, Dongli Guan from the LSU Department of Biological Sciences for assistance with PFGE.

Finally, I express my deepest appreciation to my wife, Jie Zhu for her encouragement, support, love, and great cooking, and my parents, Aihua Li and Xiliang Yan, for their consistent support and unconditional love.

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ABSTRACT

A reductively dehalogenating enrichment culture was established using chloroalkane-contaminated groundwater from the PetroProcessors of Louisiana, Inc. (PPI) Superfund site. Two novel, strictly anaerobic bacterial strains, designated as BL-DC-8 and BL-DC-9, were isolated from the enrichment culture. These strains represent the first bacteria known to anaerobically dehalogenate 1,2,3-trichloropropane, the degradation pathway of which was determined. Both strains could be cultured in completely defined basal medium and were also able to dehalogenate a variety of other vicinally chlorinated alkanes including 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,2-dichloroethane, and 1,2-dichloropropane via dichloroelimination reactions. Chlorinated alkanes containing only a single chlorine substituent (1-chloropropane, 2-chloropropane), chlorinated alkenes (tetrachloroethene, trichloroethene, 1,2-dichloroethenes, vinyl chloride), and chlorinated benzenes (1-chlorobenzene and 1,2-dichlorobenzene) were not dehalogenated. Phylogenetic analysis based on 16S rRNA gene sequences showed these isolates to represent a new lineage within the *Chloroflexi*. Their closest previously cultured relatives are “Dehalococcoides” strains with 16S rRNA gene sequence similarities of only 90%. A quantitative real-time PCR (qPCR) approach targeting 16S rRNA genes indicated that both strains couple reductive dechlorination to cell growth. Growth was not observed in the absence of hydrogen (H₂) as an electron donor and a polychlorinated alkane as an electron acceptor.

Oligonucleotide primers targeting signature 16S rRNA gene sequences of the novel isolates were used in conjunction with primers targeting “Dehalococcoides” strains to investigate spatial distribution and relative abundance of dehalogenating bacteria within the dense non-aqueous phase liquid (DNAPL) source zone at the PPI site. Results revealed the presence of bacteria similar or identical to strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” strains throughout the DNAPL source zone. 16S rRNA gene copy concentrations as high as 1.88

$\pm 0.07 \times 10^6$ copies/mL groundwater for the isolates and $5.84 \pm 0.20 \times 10^5$ copies/mL for “Dehalococcoides” strains. The new genus represented up to 18.6% of total bacterial 16S rRNA gene copies at some locations, and it may play an important role in natural attenuation at this site. These results have the potential to improve decision making regarding remediation strategies and may aid in development of waste treatment and monitoring approaches.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Chlorinated solvents are among the most common contaminants found in groundwater throughout the United States and Europe. In recent years, microbially mediated processes have been widely recognized as being more effective than traditional methods (i.e. pump-and-treat) to restore many contaminated sites. Relatively little is known, however, about the microorganisms able to reductively dehalogenate chlorinated solvents in general and chlorinated alkanes in particular. Research reported in this dissertation was aimed at characterizing bacteria involved in anaerobic reductive dehalogenation, with the goal of broadening understanding of physiology, phylogeny, and biochemical pathways of dehalorespiring bacterial populations.

This chapter provides a literature review that includes an overview of the properties of chlorinated solvents, their presence in the environment (Section 1.2), toxicological properties (Section 1.3), techniques for remediation (Section 1.4), and biotransformation processes (Section 1.5). Research objectives are presented in Section 1.6. The organization of the remaining dissertation chapters is outlined in Section 1.7.

1.2 History and Overview

Chlorinated solvents represent a variety of C₁ to C₄ aliphatic hydrocarbons and benzenes containing chlorine atom(s) in their molecular structures. Since their first introduction into industry as machine degreasers in the 1940's, over the last century, chlorinated solvents have been extensively used as organic solvents, dry cleaning agents, chemical intermediates and pesticides in industrial and agricultural processes (Sutherson, 1997; De Wildeman *et al.*, 2003; Bowman *et al.*, 2006). Among chlorinated solvents, the most commonly used are 1,2-dichloroethane (1,2-DCA), 1,1,1-trichloroethane (1,1,1-TCA), tetrachloroethene (PCE), trichloroethene (TCE), carbon tetrachloride, methylene chloride, and chloroform (Sutherson,

1997). The estimated productions of 1,2-DCA, PCE and TCE in the United States are 9,328,000, 110,000 and 123,000 tons/year, respectively (Field and Sierra-Alvarez, 2005).

Along with large productions and excessive use, chlorinated solvents had aroused public concerns for their adverse effects on the offsprings of both human and wildlife based on scientific evidence (Hileman, 1993). Some chlorinated solvents, for example, carbon tetrachloride and 1,1,1-TCA, were also identified as ozone depletion chemicals and were prohibited under the Montreal Protocol in early 1996 (Hileman, 1993; Doherty, 2000). Production and use of chlorinated solvents had been gradually phased out since the 1990's in response to these negative impacts (Hileman, 1993). Unfortunately, improper disposal and accidental spills have led to widespread contamination of chlorinated solvents in soil and groundwater that poses a threat to public health and clean water supply (Ensley, 1991; Pankow and Cherry, 1996; McCarty, 1997). Across the United States, it was reported that about 400,000 sites had been contaminated with chlorinated solvents (Sutfin, 1996). U.S. Environmental Protection Agency (EPA) estimated that from 1998 to 2001 the total releases of 1,2-DCA, PCE and TCE to water and subsurface were 1,909, 671 and 254 tons, respectively, including surface water discharges, underground injection and on-site releases to land (U.S. EPA Toxics Release Inventory Database).

Out of the 25 most frequent groundwater contaminants at the hazardous waste sites, 10 have been identified as chlorinated solvents, with TCE being the most prevalent constituent (National Research Council, 1994). PCE and TCE have been found in at least 771 and 861 of the 1430 current or former National Priorities List (NPL) sites, respectively. 1,2-DCA is found at 570 sites; 1,1,1-TCA is found at 823 sites; 1,1-DCA is found at 248 sites. The less-chlorinated ethenes, dichloroethene isomers (DCEs) and vinyl chloride are also widespread, resulting from both improper disposal and microbial transformation of PCE or TCE. *Cis*-1,2-DCE was found in

at least 146 sites and *trans*-1,2-DCE was found in at least 563 sites. At 336 of the NPL sites, 1,2-DCE was found but the isomer was not specified. The most toxic intermediate, vinyl chloride, was found in at least 662 NPL sites (Agency for Toxic Substances & Disease Registry).

1.3 Toxicological Properties

Chlorinated solvents have aroused concerns because of their toxic properties and persistence in aqueous and soil environments (Chapelle, 1999; Bradley, 2003; De Wildeman and Verstraete, 2003). Under reductive conditions, chlorinated solvents have long half-lives which are often found more than 1 year or even decades after release (Vogel *et al.*, 1987; De Wildeman and Verstraete, 2003). 1,1,2-TCA, a hazardous compound found in at least 45 NPL site, has a half-life as high as 170 years (Vogel *et al.*, 1987). Such stability has made chlorinated solvents a long term threaten to public health and wildlife.

Most of the chlorinated solvents are skin and eye irritants and can cause non-cancerous adverse effects in endocrine, immune and central nervous systems of humans, wildlife and their offspring at very low exposure levels (Hileman, 1993). For some widely used chlorinated solvents, studies also have demonstrated significantly increased incidence of liver or kidney tumors in mice or rats, and the damage of central nervous system by acute exposure via inhalation of PCE, TCE, 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), 1,1,2-TCA, 1,2-DCA and 1,2-dichloropropane (1,2-DCP) (U.S. EPA air toxics database; Agency for Toxic Substances & Disease Registry). Based on epidemiological occupational data and animal studies, these toxic chlorinated compounds have been classified as possible or probable human carcinogens by U.S.EPA (Table 1.1).

Vinyl chloride, a chemical intermediate mostly used to make polyvinyl chloride (PVC) plastic and vinyl products, was classified as Group A, a known human carcinogen, for direct evidence of inducing the risk of a rare form of liver cancer in humans. Acute exposure of

humans to high levels of vinyl chloride via inhalation can cause loss of consciousness, lung and kidney irritation, and inhibition of blood clotting (U.S. EPA air toxics database; Agency for Toxic Substances & Disease Registry).

Table 1.1. Physical properties and toxicological classifications of selected chlorinated solvents

Compound	Molecular Weight	Density ^a (g/mL)	Solubility ^b (mg/L)	Half-life ^c	Human carcinogen/ Classification ^d
PCE	165.83	1.623	200	0.7-6.0	Probable/possible Group B/C
TCE	131.4	1.463	1100	0.9-2.5	Probable
Vinyl chloride	62.5	0.911	8800	-	Confirmed Group A
1,1,2,2-TeCA	167.85	1.586	2970	0.8	Possible Group C
1,1,1-TCA	133.40	1.336	1300	0.5-2.5	N/A
1,1,2-TCA	133.4	1.435	4420	170	Probable/possible Group B/C
1,2-DCA	98.96	1.256	8520	50	Probable Group B2
1,2-DCP	113	1.156	2800	-	Probable Group B2

^a: All values are taken from Sigma-Aldrich Inc. at 25 °C with purity $\geq 99\%$. The density of vinyl chloride is estimated in gas phase.

^b: All values are taken from U.S. EPA Superfund Chemical Data Matrix (SCDM) at 25 °C.

^c: The half-lives are estimated at 10 °C-20 °C from abiotic hydrolysis or dehydrohalogenation (Vogel *et al.*, 1987).

^d: The information are taken from U.S. EPA air toxics database and Agency for Toxic Substances & Disease Registry. TCE is determined as “probably carcinogenic to human” by International Agency for Research on Cancer.

1.4 Remediation Technologies

With densities greater than water and limited aqueous solubilities (Table 1.1), chlorinated solvents tend to travel downward through the vadose zone and form a dense non-aqueous-phase liquid (DNAPL) layer at the bottom of the water table when released in the environment (Pankow and Cherry, 1996; Carr *et al.*, 2000; Cope and Hughes, 2001; Christ *et al.*, 2005). Such DNAPLs can slowly dissolve into groundwater flow, thereby serving as long-lasting sources of

dissolved chlorinated solvents that may persist at concentrations higher than safe levels in the groundwater for decades after release (Mackay and Cherry, 1989).

Traditional physical and chemical technologies such as pump-and-treat technology have been implemented in many contaminated sites. In such remediation strategies, groundwater is pumped to the surface, and dissolved chlorosolvents are removed *ex situ* via air stripping or adsorption to activated carbon. Unfortunately, since the majority of DNAPL generally remains trapped in the lower-permeability aquifer media, pump-and-treat methods are often insufficient to remediate contaminated groundwater at time scales shorter than decades or even centuries (Schwille, 1988; Mackay and Cherry, 1989; Christ *et al.*, 2005).

1.5 Biodegradation and Bioavailability

1.5.1 Chlorinated Solvent Biodegradation Under Aerobic Conditions

Chlorinated solvents can be used as a carbon and energy source for growth by several genera of bacteria and fungi. Under aerobic conditions, chlorinated solvents may serve as electron donors and can be oxidized in a thermodynamically favorable process. Theoretically, for example, complete mineralization of 1 mole 1,2-dichloroethane into carbon dioxide and chlorine will release as much as 1279 kJ Gibbs free energy (Dolfing and Janssen, 1994). Many studies have demonstrated that chlorinated ethenes can be either metabolically biodegraded as primary substrates or/and co-metabolically biodegraded at the presence of high concentrations of co-substrates (Wilson and Wilson, 1985; McCarty and Semprini, 1994; Bradley, 2003; Christ *et al.*, 2005). The potential for chlorinated ethenes to be oxidized is largely dictated by the number of chlorine atoms in their structures (Vogel *et al.*, 1987; Bradley, 2003). Vinyl chloride and DCEs, the least-chlorinated chloroethenes, have the greatest tendency to be oxidized as the primary substrates by microorganisms (Hartmans *et al.*, 1992; Verce *et al.*, 2000; Bradley and Chapelle, 2000). On the other hand, PCE and TCE, the most-chlorinated chloroethenes, are extremely

difficult to break up under aerobic conditions because of their relatively high oxidation states. Studies have demonstrated that TCE can be fortuitously oxidized by methanotrophic organisms when supplied with high concentrations of methane (Wilson and Wilson, 1985; McCarty and Semprini, 1994; Semprini, 1995). So far, there is only one report that PCE can be aerobically oxidized in a co-metabolic manner by a wastewater isolate, *Pseudomonas stutzeri* strain OX1, at the presence of *o*-xylene, toluene, cresols, 2,3-dimethylphenol, or 3,4-dimethylphenol as sole carbon and energy source (Ryoo *et al.*, 2000; Shim *et al.*, 2001).

Aerobic biodegradation, unfortunately, is often unsuitable for *in-situ* bioremediation applications. Oxygen, the electron acceptor required in this process, is usually absent or present at very low concentrations in subsurface environments. Effective transfer oxygen into the underground is still questionable due to the high pumping cost and unwanted loss through reaction with reduced species such as Fe^{2+} , S^{2-} . Although some chlorinated solvents can be utilized as the primary substrates, many others, such as PCE and TCE, are poor choices as microbial growth substrates under aerobic conditions. Their oxidation requires continuous supply of high concentrations of primary substrates for co-metabolic processes, which is often unacceptable or undesirable for achieving long-term remediation goals from an engineering perspective.

1.5.2 Chlorinated Solvent Biodegradation Under Anaerobic Conditions

In the early 1980's, observations of reduced daughter products at several contaminated sites stimulated the study on anaerobic biodegradation of chlorinated solvents (Barrio-Large *et al.*, 1987; Bouwer, 1994; McCarty and Semprini, 1994). In addition to serve as reductants, chlorinated compounds also act as strong oxidizers in low redox potential environments. At the presence of suitable electron donors (i.e. hydrogen or acetate), chlorinated compounds can be reduced as terminal electron acceptors to generate energy for microbial growth. This is

commonly referred to as “reductive dechlorination”, also known as halorespiration (McCarty, 1997). In this metabolic process, chlorinated compounds are sequentially transformed by having chlorine atoms substituted with hydrogen atoms transferred from an electron donor.

In contrast to aerobic oxidation, the more-chlorinated chloroethenes have greater tendency to undergo reductive dechlorination reactions under anaerobic conditions (Vogel *et al.*, 1987; Bradley, 2003). PCE, the most recalcitrant compound under aerobic oxidation, turns out to be one of the most favorable electron acceptors under anaerobic conditions. For example, PCE can be completely dehalogenated via the pathway involving sequential transformation to TCE, DCE, VC, and finally the non-toxic end product ethene (Maymo-Gatell *et al.*, 1997). Thermodynamic calculations indicate that free energy may be released from every step of PCE dechlorination and can therefore be potentially captured by microorganisms to support growth (Table 1.2). Besides PCE, many of the other commonly occurring chlorinated contaminants (i.e. TCE, DCE isomers, 1,1,2-TCA, 1,1,1-TCA, 1,2-DCA, 1,2-DCP, vinyl chloride and some chlorobenzenes) are also can be utilized as electron acceptors to support microbial growth in pure or mixed cultures (Maymo-Gatell *et al.*, 1997; Maymo-Gatell *et al.*, 1999; Adrian *et al.*, 2000a, 2000b; Sun *et al.*, 2002; Cupples *et al.*, 2003; De Wildeman *et al.*, 2003; He *et al.*, 2003; Ritalahti and Löffler, 2004; He *et al.*, 2005; Sung *et al.*, 2006). Reductive dechlorination of TCE to *cis*-1,2-DCE was observed under Fe (III) reduced conditions and more reducing environments (Chapelle, 1996; Bradley, 2003). Under sulfate-reducing and methanogenic conditions, *cis*-1,2-DCE is more likely to be dehalogenated into vinyl chloride (Chapelle, 1996; Bradley, 2003). However, the reductive dechlorination of vinyl chloride to ethene is more rare and appears to be more slow and incomplete, and normally requires higher reducing conditions (Ballapragada *et al.*, 1995; Bouwer, 1994; Carter and Jewell, 1993; Freedman and Gossett, 1995; Wu *et al.*, 1995; Bradley, 2003).

Table 1.2. Gibbs free energy for selected reductive dechlorination processes

Chlorinated compounds	End product	Half reactions	ΔG_f^0 , kJ/eeq ^a
PCE	TCE	$\frac{1}{2} \text{C}_2\text{Cl}_4 + \frac{1}{2} \text{H}^+ + \text{e}^- = \frac{1}{2} \text{C}_2\text{HCl}_3 + \frac{1}{2} \text{Cl}^-$	-55.3
TCE	<i>Cis</i> -1,2-DCE	$\frac{1}{2} \text{C}_2\text{HCl}_3 + \frac{1}{2} \text{H}^+ + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_2\text{Cl}_2 + \frac{1}{2} \text{Cl}^-$	-53.0
<i>Cis</i> -1,2-DCE	Vinyl chloride	$\frac{1}{2} \text{C}_2\text{H}_2\text{Cl}_2 + \frac{1}{2} \text{H}^+ + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_3\text{Cl} + \frac{1}{2} \text{Cl}^-$	-38.3
Vinyl chloride	Ethene	$\frac{1}{2} \text{C}_2\text{H}_3\text{Cl} + \frac{1}{2} \text{H}^+ + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_4 + \frac{1}{2} \text{Cl}^-$	-43.5
1,1,2-TCA	Vinyl chloride	$\frac{1}{2} \text{C}_2\text{H}_3\text{Cl}_3 + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_3\text{Cl} + \text{Cl}^-$	-78.1
1,2-DCA	Ethene	$\frac{1}{2} \text{C}_2\text{H}_4\text{Cl}_2 + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_4 + \text{Cl}^-$	-71.4
1,1,2,2-TeCA	<i>Cis</i> -1,2-DCE	$\frac{1}{2} \text{C}_2\text{H}_2\text{Cl}_4 + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_2\text{Cl}_2 + \text{Cl}^-$	-90.4
	<i>Trans</i> -1,2-DCE	$\frac{1}{2} \text{C}_2\text{H}_2\text{Cl}_4 + \text{e}^- = \frac{1}{2} \text{C}_2\text{H}_2\text{Cl}_2 + \text{Cl}^-$	-88.0
1,2-DCP	Propene	$\frac{1}{2} \text{C}_3\text{H}_6\text{Cl}_2 + \text{e}^- = \frac{1}{2} \text{C}_3\text{H}_6 + \text{Cl}^-$	-69.6

^a: Values are calculated from Vogel *et al.*, 1987, Dolfing and Jensen, 1994 and De Wildeman and Verstraete, 2003.

The promise of anaerobic reductive dechlorination to address problems associated with chlorinated solvent contamination has triggered the search for dehalorespiring microbial populations. Several genera of anaerobic bacteria including *Desulfonomonile*, *Dehalobacter*, *Desulfuromonas*, *Geobacter*, *Sulfurospirillum*, *Desulfitobacterium*, *Clostridium*, *Desulfovibrio*, *Enterobacter*, *Anaeromyxobacter* and “Dehalococcoides” have been identified as able to carry out reductive dehalogenation of a variety of chlorinated solvents (Shelton and Tiedje, 1984; Holliger *et al.*, 1993; Löffler *et al.*, 1999, 2000, 2003; Bradley, 2003).

Desulfonomonile tiedjei strain DCB-1, a sulfate reducing bacteria, was found able to couple reductive dechlorination of 3-chlorobenzoate to growth and also capable of dehalogenating PCE into TCE and DCE through dehalorespiration (Shelton and Tiedje, 1984;

Deweerd and Suflita, 1990; Cole *et al.*, 1995). Later, a number of microbial pure cultures capable of coupling growth to the dehalogenation of PCE and TCE to DCE were recovered from contaminated environments, including *Dehalobacter restrictus*, *Desulfitobacterium* strain PCE-1, *Desulfuromonas chloroethenica*, *Desulfuromonas* strain BB1, *Enterobacter agglomerans* strain MS-1 (Holliger *et al.*, 1993; Gerritse *et al.*, 1996; Löffler *et al.*, 2000, 2003; Krumholz *et al.*, 1996, 1997; Sharma and McCarty, 1996).

Among these dehalorespiring populations, “Dehalococcoides” strains have received a great deal of attention in recent years due to the unique capacity to completely detoxifying PCE and other chlorinated ethenes into the non-toxic final product of ethene. (Maymo-Gatell *et al.*, 1997, 2001; Cupples *et al.*, 2003; He *et al.*, 2003a, 2005; Duhamel *et al.*, 2004; Sung *et al.*, 2006). “Dehalococcoides” strains are only known to grow when supplied with appropriate chlorinated solvents as terminal electron acceptors and hydrogen as electron donor. Strains previously isolated in pure culture are all morphologically similar and share more than 98% similarity in 16S rRNA gene sequences (Maymo-Gatell *et al.*, 1997, 1999, 2001; Adrian *et al.*, 2000a; He *et al.*, 2003a; Ritalahti and Löffler, 2004; He *et al.*, 2005).

“Dehalococcoides ethenogenes” strain 195 was the first strain isolated within this group. So far, strain 195 was also the only known organism able to completely detoxify PCE all the way down into non-toxic product of ethene. Growth of strain 195 was found to be coupled with each dehalogenation step, except the last one from vinyl chloride to ethene, which was a slow and incomplete co-metabolic process (Maymo-Gatell *et al.*, 1997, 2001). Besides the chlorinated ethenes, 1,2-DCA and 1,2-dibromoethane could also be utilized by strain 195 and the final products were ethene (Maymo-Gatell *et al.*, 1997, 1999).

“Dehalococcoides” strain CBDB1 was the first bacterium demonstrated in pure culture to link growth with dehalogenation of chlorinated benzenes (Adrian *et al.*, 2000a, 2000b). 1,2,3-

Trichlorobenzene (1,2,3-TCB), 1,2,4-TCB, 1,2,3,4-tetrachlorobenzene (1,2,3,4-TeCB), 1,2,3,5-TeCB and 1,2,4,5-TeCB could be stoichiometrically dehalogenated to dichlorobenzenes or 1,3,5-TCB. Strain CBDB1 was also found to be capable of reductively dehalogenating selected dioxin congeners but not chlorinated ethenes (e.g. PCE and TCE) (Bunge *et al.*, 2003). Dehalogenation of chlorinated aromatic compounds was also found to be carried out by “Dehalococcoides” strain 195 in a follow up study (Fennell *et al.*, 2004).

Vinyl chloride dechlorinators have been reported in several mixed cultures and the responsible organisms were identified as “Dehalococcoides”-like strains (He *et al.*, 2003b; Cupples *et al.*, 2003; Duhamel *et al.*, 2004). This crucial step for the complete detoxification of chloroethenes was finally resolved as “Dehalococcoides” strain BAV1 was isolated. Besides vinyl chloride, this organism also coupled growth with vinyl bromide, 1,2-DCA and less-chlorinated chloroethenes including *cis*- and *trans*-1,2-DCE. PCE and TCE were dehalogenated only co-metabolically by strain BAV1 (He *et al.*, 2003a). Later on, two more isolates, strains FL2 and GT were added in the “Dehalococcoides” group. Strain FL2 linked cell growth with dehalogenation of TCE, *cis*-1,2-DCE and *trans*-1,2-DCE, and co-metabolically dehalogenated PCE and vinyl chloride (He *et al.*, 2005). Strain GT was the second pure culture isolate that dehalogenated vinyl chloride into ethene metabolically; it could also dehalogenate TCE, *cis*-1,2-DCE, 1,1-DCE but not PCE (Sung *et al.*, 2006).

The availability of a suitable electron donor is crucial for accomplishing reductive dechlorination. For pure cultures, hydrogen (H₂) is generally considered to be the ultimate and only electron donor for *Dehalobacter* and “Dehalococcoides” strains (Holliger *et al.*, 1993; Löffler *et al.*, 1999, 2000; Sun *et al.*, 2002; He *et al.*, 2002; Bradley, 2003). In contrast, *Desulfuromonas* strains are believed to require acetate instead of hydrogen to complete dechlorination (Krumholz *et al.*, 1996, 1997; Bradley, 2003). In studies employing mixed

cultures, it has been observed that compounds, such as methanol, formate, lactate, butyrate propionate, benzoate and glucose, can be fermented by syntrophic microorganisms into hydrogen or acetate which then can be consumed as terminal electron donors (Ballapragada *et al.*, 1997; Yang and McCarty, 1998; Fennell *et al.*, 2001; Bradley, 2003).

Numerous efforts have been made to comprehensively understand the dehalogenation of chlorinated alkenes out of the major concerns on the widespread contaminations caused by PCE and TCE. Much less information, however, is available regarding reductive dehalogenation of chlorinated alkanes (e.g. chlorinated ethanes and propanes); the microbial populations capable of utilizing them as carbon or energy source remain largely unknown.

Many chlorinated alkanes have no known aerobic microbial pathways or can only be oxidized in an incomplete and slow co-metabolic manner, and effective transfer of oxygen into subsurface is also practically difficult (Löffler *et al.*, 1997; Ritalahti and Löffler, 2004; De Wildeman and Verstraete, 2003; Field and Sierra-Alvarez, 2004). Under anaerobic conditions, several chlorinated alkanes may be utilized as terminal electron acceptors to support microbial growth in halorespiration processes similar to what has been observed for chlorinated ethenes.

Dehalobacter strain TCA1 is the only known isolate able to dehalogenate 1,1,1-TCA in a growth-linked process (Sun *et al.*, 2002). With H₂ or formate as the electron donor, 1,1,1-TCA was sequentially converted into 1,1-DCA which was further dehalogenated into monochloroethane. Other chlorinated alkanes, including 1,1,1,2-TeCA, 1,1,2-TCA, 1,2-DCA and 1,2-DCP, and some chloroethenes (e.g. PCE and TCE), however, were not dechlorinated by strain TCA1.

Many polychlorinated alkanes, such as 1,2-DCA, 1,1,2-TCA, 1,1,2,2-TeCA and 1,2-DCP, can also be grouped into vicinally chlorinated alkanes, where two or more chlorines are bonded to two adjacent carbon atoms. The dihaloelimination reactions appear to be the

thermodynamically most favorable reductive dechlorination pathway for these compounds. In the hydrogenolysis reaction, as observed for 1,1,1-TCA and most chloroethenes, one chloride atom was substituted by hydrogen atom with the net input of two electrons at each dehalogenation step. In the dihaloelimination reaction, however, two chlorines are removed from adjacent carbon atoms and a double bond is formed instead of introducing hydrogen atom into its structure (De Wildeman and Verstraete, 2003).

“Dehalococcoides ethenogenes” strain 195 was reported to partially dehalogenate 1,2-DCA into ethene with up to 1% accumulation of vinyl chloride. It indicated that vinyl chloride could be the intermediate of 1,2-DCA dehalogenation, and thus the dihaloelimination capacity of strain 195 was still questionable (Maymo-Gatell *et al.*, 1997, 1999).

A few studies conducted in mixed cultures found that as yet uncharacterized *Dehalobacter* and “Dehalococcoides” strains contributed to the dihaloelimination reaction of 1,2-DCP to propene (Löffler *et al.*, 1997; Ritalahti and Löffler, 2004; Schlotelburg *et al.*, 2000, 2002). Grostern and Edwards demonstrated that bacteria belonging to *Dehalobacter* were responsible for the dehalogenation step of 1,1,2-TCA directly to vinyl chloride, but not vinyl chloride to ethene, in a mixed culture capable of dehalogenating 1,1,2-TCA and 1,2-DCA to ethene (Grostern and Edwards, 2006). This dihaloelimination reaction was also observed in the dehalogenation of 1,1,2,2-TeCA to 1,2-DCEs by wetland sediments, and the dechlorinators were tentatively linked to “Dehalococcoides” and *Desulfuromonas* strains. However, no pure cultures were recovered from these studies.

Desulfitobacterium dichloroeliminans strain DCA1 is the only isolate known to exclusively dihaloeliminate vicinally chlorinated ethanes (De Wildeman *et al.*, 2003). Compounds serving as electron acceptors included 1,2-DCA, 1,1,2-TCA, 1,2-DCP and some vicinally chlorinated butanes. Strain Y51, another isolate from the genus *Desulfitobacterium*, had

a wide range of dihaloelimination capacities for hexachloroethane, pentachloroethane, TeCA and 1,1,1,2,2,3,3-heptachloropropane. It is interesting to note that strain Y51 also dehalogenated PCE and TCE into *cis*-1,2-DCE (Suyama *et al.*, 2001). All these evidences concluded that community of chloroalkane-dechlorinators could possibly be as diverse as that of chloroethene-dechlorinators, but their ecology, phylogeny and physiology are still poorly understood. This limits the development of viable biological processes to remediate sites contaminated by chlorinated alkanes.

1.6 Research Objectives

The overall objective of this research was to isolate and characterize dehalogenating bacteria and provide a more comprehensive understanding of the microbial populations responsible for reductive dehalogenation of chlorinated solvents in general and chlorinated alkanes in particular.

1.7 Dissertation Organization

Chapter 2 describes microcosm experiments used to establishment of a 1,1,2-TCA dehalogenating enrichment culture using contaminated groundwater at the PetroProcessors of Louisiana, Inc. (PPI) Superfund site located near Baton Rouge, LA, as the inoculum. It also describes isolation of reductively dechlorinating bacteria from the enrichment culture. Chapter 3 presents the characterization of the novel dehalogenation bacteria. Chapter 4 describes studies conducted to assess the abilities of two novel isolates to reductively dehalogenate 1,2,3-trichloropropane. The anaerobic biodegradation pathway for 1,2,3-trichloropropane is also presented. Chapter 5 describes development and application of DNA-based techniques for detecting and quantifying the novel bacteria. Chapter 6 summarizes the major conclusions from the research and makes recommendations for the future work. Chapter 7 contains a listing of references cited throughout the dissertation.

CHAPTER 2. ENRICHMENT AND ISOLATION OF ANAEROBIC REDUCTIVE DECHLORINATION BACTERIA FROM A SUPERFUND SITE

2.1 Introduction

As described in Chapter 1, the microbial populations able to dehalogenate chlorinated alkanes remain largely unknown. In this study, investigation of reductively dechlorinating bacterial populations was initiated by constructing anaerobic enrichment cultures seeded with groundwater from the PetroProcessors of Louisiana, Inc. (PPI) Superfund Site located near Baton Rouge, Louisiana. From 1969 to 1980, petrochemical wastes, including free-phase chlorinated solvents, were disposed at this site by direct discharge to unlined earthen ponds (Clement *et al.*, 2002). Groundwater in the DNAPL source zone remaining at the site is contaminated with high concentrations of a variety of chlorinated alkanes including 1,2-DCA, 1,1,2-TCA, 1,2-DCP, and 1,1,2,2-TeCA (Bowman *et al.*, 2006). In a recent study, it was reported that the dehalogenation daughter products vinyl chloride and ethene were detected at high aqueous-phase concentrations (52.2 and 9.57 mg/L, respectively) within the DNAPL source zone groundwater, and microbial cell concentrations greater than 3×10^7 cells/mL were determined in the groundwater based on direct counting via microscopy (Bowman *et al.*, 2006). The presence of dechlorination products and high microbial cell concentrations suggested that dehalogenation processes are likely occurring within the DNAPL source zone.

In experiments described in this chapter, anaerobic enrichment cultures were established followed by a dilution-to-extinction approach for the isolation of dehalogenating bacteria.

2.2 Materials and Methods

2.2.1 Chemicals

1,1,2-Trichloroethane (1,1,2-TCA, 98%) and 1,2-dichloroethane (1,2-DCA, >99.9%) were purchased from Sigma-Aldrich. Certified gas standards for vinyl chloride (1002 ppm in

nitrogen), and ethene (99.5 %) were purchased from Supelco (St. Louis, MO).

2.2.2 Groundwater Collection

The groundwater sample used for construction of enrichment cultures was collected from a waste recovery well (well ID no. W-1024) located in the DNAPL source zone at the Brooklawn portion of the PPI Superfund site in March 2005. Prior to sample collection, the well was purged, with more than three well volumes pumped before sample collection. Groundwater was collected in a sterile 1.0 L glass bottles leaving little or no headspace, and sample was placed in a cooler on ice during transport to the laboratory (approximately one hour).

2.2.3 Enrichment Culture

The groundwater from the PPI site was purged with filter-sterilized ultra high purity nitrogen (N₂) gas for one hour to volatilize chlorosolvents present at the time of collection. Then, working inside an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI), 30 mL aliquots of groundwater were inoculated into 550 mL serum bottles containing 300 mL of anaerobic basal medium.

The anaerobic basal medium contained the following (per liter) constituents: NaCl, 1.0 g; MgCl₂·6H₂O, 0.4 g; CaCl₂·6H₂O, 0.1 g; KH₂PO₄, 0.2 g; NH₄Cl, 0.25 g; KCl, 0.5 g; resazurin, 0.001 g; L-cysteine hydrochloride, 0.25 g; sodium acetate 0.41 g (5 mmol); sodium pyruvate 0.55 g (5 mmol); sodium lactate 60% (m/m) syrup, 0.7 mL (5 mmol); non-chelated trace element solution, 1.0 mL (Kuever *et al.*, 2005); and 1.0 mL selenite-tungstate solution (Alain *et al.*, 2002). After the medium was autoclaved under an N₂ atmosphere and cooled, the following components were added aseptically from sterile stock solutions: 30 mL of NaHCO₃ solution (84 g/L autoclaved under N₂ atmosphere), 7.5 mL of Na₂S solution (48 g/L Na₂S·9H₂O autoclaved in sealed brown bottle), 0.1 mL vitamin solution (40 mg/L 4-aminobenzoic acid, 10 mg/L D(+)-biotin, 100 mg/L nicotinic acid, 50 mg/L calcium D (+)-pantothenate, 150 mg/L pyridoxine

dihydrochloride, filter-sterilized and stored in 10 mM pH 7.1 sodium phosphate buffer at 4 °C), 0.1 mL thiamin solution (1 g/L thiamine chloride, filter-sterilized and stored in 25 mM pH 3.4 sodium phosphate buffer at 4 °C), 0.1 mL vitamin B₁₂ solution (500 mg/L, filter-sterilized and stored in distilled water at 4 °C). The pH of the medium was adjusted to 7.0-7.5 with 2.4 M HCl (filter-sterilized). The medium was then dispensed aseptically into sterile serum bottles and sealed with butyl-rubber stoppers and aluminum crimp caps. Gas headspace of the serum bottles was flushed with a filter-sterilized H₂/CO₂/N₂ gas mixture (10%/10%/80%, v/v/v).

Each bottle was spiked with 14 µL of filter-sterilized neat 1,1,2-TCA to reach a final aqueous-phase concentration of approximately 0.5 mM (60 mg/L). Unless indicated otherwise, cultures were incubated under static conditions in the dark at a temperature of 30°C. Replicate bottles were analyzed via gas chromatography at weekly intervals during a three week incubation period. Subsequent transfers were made when 1,1,2-TCA was completely transformed into ethene. The 1,1,2-TCA enrichment culture was then maintained with low carbon substrates (0.05 mM acetate, 0.05 mM pyruvate and 0.05 mM lactate) after two transfers.

An additional enrichment culture was constructed but with 1,2-DCA amended as the electron acceptor. The composition and preparation of defined basal medium were the same as described above, except that only acetate (5 mM) was provided as carbon source. The 1,2-DCA enrichment culture was maintained in the dark at 30 °C and analyzed for 1,2-DCA dechlorination at weekly intervals. Subsequent transfers were made when 1,1,2-TCA was completely transformed into ethene.

2.2.4 Bacterial Isolation

In a dilution-to-extinction procedure, the 1,1,2-TCA-degrading enrichment culture was serially diluted 10⁻³ to 10⁻⁸ using the anaerobic basal medium, 1,1,2-TCA concentrations, and incubation conditions identical to those used in the enrichment microcosm but with 15 mL liquid

media in 25 mL volume serum bottles, 5 mM sodium acetate as carbon source (no pyruvate or lactate were added), and addition of ampicillin (1g/L) and vancomycin (0.1g/L) as selective agents.

25 replicate serum bottles were set up at each dilution level. After two months incubation, 1.0 mL was sampled from each bottle and analyzed for dechlorination activities. Detection of vinyl chloride or other dechlorination products was used as the indicator for scoring bottles as positive for dechlorination of 1,1,2-TCA. Bottles in which no dechlorination daughter products were detected were scored as negative.

2.2.5 DNA Extraction and 16S rRNA Gene Sequencing

Biomass was collected from each dechlorination positive bottle in the 10^{-4} to 10^{-8} dilution levels by centrifuging 10 mL culture at $8,200\times g$ for 15 min. Prior to cell collection, 1 mL of culture from each positive bottle was transferred to a bottle containing fresh basal medium amended with 1,1,2-TCA to allow propagation.

Bacterial DNA was extracted from 10 mL of culture and purified using an UltraClean Microbial DNA Isolation kit (MioBio Laboratories Inc., Carlsbad, CA). The 16S rRNA gene was amplified using universal bacterial primers 27f and 1525r as described by Rainey *et al.* (1996). Then, 16S rRNA gene products were sequenced with the forward primers 27f and 536f. Sequences were manually inspected using the BioEdit software package. For sequences that appeared likely to originate from pure cultures, nearly complete 16S rRNA gene sequences (comprising approximately 1300 nucleotide positions) assembled using BioEdit were analyzed using the BLAST interface of the National Center for Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/>). The 16S rRNA gene sequences from nine putative pure bacterial strains isolated from the enrichment culture were subsequently aligned by Brian Rash using the ARB software package (Ludwig *et al.*, 2004).

2.2.6 Analytical Methods

Quantitative analysis of 1,1,2-TCA, 1,2-DCA, vinyl chloride, and ethene was performed using an HP model 6890 gas chromatograph equipped with a flame ionization detector (FID) and GS-GasPro capillary column (60 m × 0.32 mm I.D., J&W P/N 113-4362). The temperature program was 80 °C for 0.5 min, increased at 25 °C/min to 175 °C and held for 2 min, and then increased at 25 °C/min to 250 °C and held for 8 min. Helium was used as carrier gas with a flowrate of 40 cm/sec (3.3 ml/min). For gas headspace samples, 100 µL gas was introduced to the GC via direct, splitless injection using gas-tight glass syringes. For aqueous-phase samples, 1.0 mL aliquots were purged with helium in a Tekmar 3000 Purge and Trap for 15 min at a flowrate of 150 mL/min. Chlorinated compounds were concentrated in the activated carbon trap of a Tekmar 2016 concentrator and delivered into GC for analysis.

Standard solutions were prepared by dissolving known volume aliquots of neat chlorinated compounds into 100 mL water in sealed serum bottles. After solutions were mixed and allowed to equilibrate, 1.0 mL liquid volumes were loaded into the purge and trap autosampler and analyzed as described above. Serial dilutions of ethene were made by injecting known volumes of ethane gas into sealed glass serum bottles containing ultra high purity N₂ and the diluted gas samples analyzed by direct injection. Calibration curves were prepared by plotting peak area against molar concentration.

2.3 Results

2.3.1 Construction of Dehalogenation Enrichment Culture

Anaerobic, sediment-free, reductively dehalogenating enrichment cultures were successfully established using 1,2-DCA or 1,1,2-TCA as electron acceptors. In the 1,2-DCA microcosms, 1,2-DCA was completely dehalogenated to ethene within four weeks with no intermediate products (e.g., monochloroethane) observed (data not shown). In the 1,1,2-TCA

microcosm, dehalogenation of 1,1,2-TCA started in this first week with vinyl chloride observed as the only daughter product. Essentially complete stoichiometric transformation of the 1,1,2-TCA to the intermediate vinyl chloride was observed by the end of the second week. More than 60% of the vinyl chloride was sequentially transformed to ethene by the end of the third week (Fig. 2.1). No methane formation was detected along with the dehalogenation of 1,1,2-TCA or 1,2-DCA.

After the dehalogenation was completed, the enrichment cultures were transferred following the same protocol. Subsequent transfers were made when 1,1,2-TCA or 1,2-DCA was completely dehalogenated into ethene in the previous culture. The 1,1,2-TCA and 1,2-DCA dehalogenating enrichment cultures have been steadily maintained in the completely defined basal medium for more than 3 years.

2.3.2 Isolation of Dehalogenating Bacteria and Phylogenetic Analysis

In the dilution-to-extinction experiment starting with the 1,1,2-TCA dehalogenating enrichment culture, a total of 61 bottles in the 10^{-3} to 10^{-6} dilution series exhibited production of vinyl chloride and were scored as dechlorination positive after two months of incubation (Table 2.1). No positive bottles was identified in the 10^{-7} and 10^{-8} dilutions (i.e., no vinyl chloride or other dechlorination daughter products were detected).

DNA was extracted and sequenced from cell material from the 36 positive bottles in the 10^{-4} , 10^{-5} and 10^{-6} dilution series. Of these, nine were tentatively identified as putative pure cultures following visual inspection of sequences using BioEdit (Table 2.2). BLAST analyses conducted using approximate 1300 nucleotide positions of the 16S rRNA gene sequences from the nine putative pure cultures indicated that seven of the putative isolates, designated as BL-DHC-1 to BL-DHC-7, were found to be closely related to the previously reported “Dehalococcoides” strains 195 and VS, with 16S rRNA gene sequence similarities ranging from

98.5% to 99.7%.

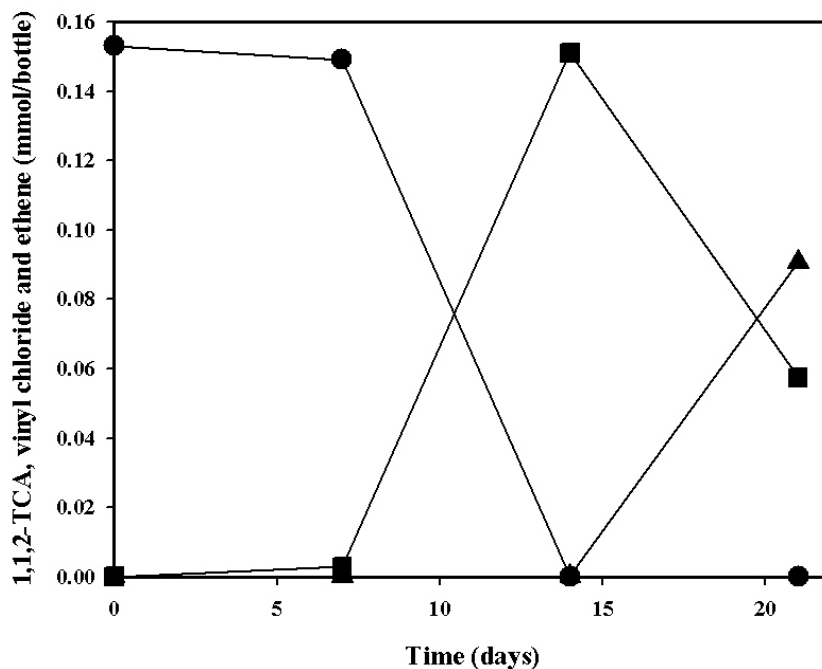


Figure 2.1. Dehalogenation of 1,1,2-TCA in an anaerobic enrichment culture. (●) 1,1,2-TCA; (■) Vinyl chloride; (▲) Ethene.

These “Dehalococcoides” strains were also found to be distantly related to the other two putative isolates, BL-DC-8 and BL-DC-9, but with only 90% similarities (Table 2.2). These nine isolates were preserved in anaerobic basal medium amended with 5% dimethyl sulfoxide (DMSO) and stored at -80 °C for future study.

Table 2.1. Dechlorination positive bottles in the dilution-to-extinction series

Dilution level	Positive	Negative
10^{-3}	25	0
10^{-4}	22	3
10^{-5}	8	17
10^{-6}	6	19
10^{-7}	0	25
10^{-8}	0	25

2.4 Discussion

In a previous study on characterization of bacterial populations in the DNAPL source zone at the PPI Superfund Site, Bowman *et al.* (2006) inferred that natural attenuation in the DNAPL source zone was likely occurring based on high microbial concentration ($>3 \times 10^7$ cells/mL), presence of dechlorination daughter products (i.e. vinyl chloride, ethene and ethane), and detection of 16S rRNA gene sequences sharing high similarity to known dehalogenating bacteria (e.g., “Dehalococcoides” strains). Successful establishment of 1,1,2-TCA and 1,2-DCA dechlorinating enrichment cultures described in this chapter experimentally further supports the earlier general conclusion by demonstrating that viable anaerobic reductively dechlorinating microbial populations are present in the DNAPL source zone of the PPI site.

Nine putative dechlorinating pure cultures were obtained from the 1,1,2-TCA enrichment culture. Of these, seven, each with a unique 16S rRNA gene sequence, were found to be highly related but not identical to previously “Dehalococcoides” strains. These results further indicate the presence of and some degree of diversity among the “Dehalococcoides” group in this DNAPL source zone. This finding is consistent with the previous “Dehalococcoides” clone library study in which 62 unique 16S rRNA gene sequences were found with high similarities (ranging from 99.5% to 99.8%) to previously reported “Dehalococcoides ethenogenes” strain 195 (Bowman *et al.*, 2006).

Two putative dehalogenating isolates recovered in this study, strains BL-DC-8 and BL-DC-9, were found to be distantly related to “Dehalococcoides” strains on the basis of low similarity in 16S rRNA gene sequences. This indicated that they represented novel as yet uncharacterized dechlorinating organisms that may play an important role in natural attenuation in the DNAPL source zone at the PPI site. Experiments conducted to establish culture purity and characterize BL-DC-8 and BL-DC-9 are presented in chapters 3 to 6.

Table 2.2. 16S rRNA gene phylogenetic analysis of the putative pure cultures isolates

Strain designation	Closest cultured phylogenetic relative (GeneBank accession number)	% Identity
BL-DHC-1	“Dehalococcoides” strain VS (AY323233)	99.5
BL-DHC-2	“Dehalococcoides ethenogenes” strain 195 (AF004928)	99.7
BL-DHC-3	“Dehalococcoides ethenogenes” strain 195 (AF004928)	98.5
BL-DHC-4	“Dehalococcoides ethenogenes” strain 195 (AF004928)	98.5
BL-DHC-5	“Dehalococcoides ethenogenes” strain 195 (AF004928)	99.5
BL-DHC-6	“Dehalococcoides ethenogenes” strain 195 (AF004928)	99.5
BL-DHC-7	“Dehalococcoides ethenogenes” strain 195 (AF004928)	99.5
BL-DC-8	“Dehalococcoides ethenogenes” strain 195 (AF004928)	90.0
BL-DC-9	“Dehalococcoides ethenogenes” strain 195 (AF004928)	90.0

Two putative dehalogenating isolates recovered in this study, strains BL-DC-8 and BL-DC-9, were found to be distantly related to “Dehalococcoides” strains on the basis of low similarity in 16S rRNA gene sequences. This indicated that they represented novel as yet uncharacterized dechlorinating organisms that may play an important role in natural attenuation in the DNAPL source zone at the PPI site. Experiments conducted to establish culture purity and characterize BL-DC-8 and BL-DC-9 are presented in chapters 3 to 6.

2.5 Conclusions

Anaerobic enrichment cultures able to reductively dehalogenate 1,1,2-TCA and 1,2-DCA were successfully established using chlorosolvent-contaminated groundwater collected from the PPI Superfund site as the inoculum. Anaerobic transformation of these two compounds in microcosm bottles confirmed previous inferences by Bowman *et al.* (2006) that viable bacteria with the ability to anaerobically biotransform chlorinated solvents were likely present within the

DNAPL source zone in the Brooklawn portion of the PPI site.

Using a dilution-to-extinction procedure, seven putative pure cultures of bacteria with high similarity (>98%) to “Dehalococcoides” strains in 16S rRNA gene sequences were recovered from the 1,1,2-TCA degrading enrichment culture. Two additional putative pure cultures somewhat related to “Dehalococcoides” strains but only distantly so (90% 16S rRNA gene sequence identity) were also recovered. Although further research beyond that presented in this chapter was necessary to establish culture purity, the data presented in this chapter supports the previous finding by Bowman *et al.* (2006) that there is appreciable diversity among the dehalogenating bacterial population at the PPI site.

CHAPTER 3. CHARACTERIZATION OF TWO NOVEL DEHALOGENATING BACTERIA

3.1 Introduction

This chapter describes experiments conducted to characterize two strains, BL-DC-8 and BL-DC-9, which were tentatively identified as putative pure cultures with the ability to reductively dehalogenate 1,1,2-TCA (see Chapter 2). Experiments were designed to comprehensively investigate culture purity, strain morphology, dehalogenation capability, electron donors, as well as some growth, chemotaxonomic, and physiological characteristics.

3.2 Material and Methods

3.2.1 Chemicals

Perchloroethene (PCE), *cis*-1,2-dichloroethene (*cis*-DCE), *trans*-1,2-dichloroethene (*trans*-DCE), 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), 1,1,2-trichloroethane (1,1,2-TCA), 1,1,1-trichloroethane (1,1,1-TCA), 1,2-dichloroethane (1,2-DCA), 1,2-dichloropropane (1,2-DCP), 1-chloropropane, 2-chloropropane, monochlorobenzene and 1,2-dichlorobenzene were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroethene (TCE) and 1,2-dichlorobenzene were purchased from Mallinckrodt Baker Co. (Phillipsburg, NJ). Certified gas standards for vinyl chloride (1002 ppm in nitrogen), ethene (99.5 %) and propene (99%) were purchased from Supelco (St. Louis, MO).

3.2.2 Culture and Growth Conditions

Strains BL-DC-8 and BL-DC-9 were grown in the defined basal medium as described in Chapter 2. Unless noted otherwise, approximately 0.5 mM 1,1,2-TCA or 1,2-DCP was amended as an electron acceptor, sodium acetate (5 mM) was supplied as a carbon source, and 10% H₂ was provided in the headspace. Unless noted otherwise, incubation conditions for experiments reported in this chapter were at 30 °C in dark without shaking. Sodium sulfide was omitted from

the culture prepared for microscopic examination to avoid difficulties associated with precipitate formation.

3.2.3 Microscopy

For scanning electron microscopy, cells were collected by filtration and fixed on a 0.2 μ m pore polycarbonate filter in 2% glutaraldehyde, 1% formaldehyde, 0.1 M cacodylate buffer, pH 7, for 60 min; rinsed with 0.1 M buffer, followed by distilled water; dehydrated with a graded ethanol series; critical point dried in a Denton DCP-1; mounted on aluminum specimen stubs and coated with gold:palladium 60:40 in an Edwards S-150 sputter coater; and imaged with a FEI Quanta 200 scanning electron microscope (SEM).

For transmission electron microscopy (TEM), cells were collected and fixed as described above. The membrane was rinsed with 0.02 M glycine in 0.1 M cacodylate buffer for 5 times in 1 hr. The membrane was post-fixed in 2% osmium tetroxide for 1 hr. Then the membrane was rinsed briefly with water and stained with 0.5% uranyl acetate for 1 hr in dark. The membrane was rinsed briefly with water again and dehydrated with ethanol series in 1 hr. The membrane was infiltrated with 1:1 ethanol: LR white embedding resin for 1 hr and infiltrated with 100% resin for another one hour. Fixed cells were scratched from the membrane and embedded in resin at 60 °C overnight. Solidified resin was sectioned with DuPont MT5000 or Sorvall MT or MT2 ultramicrotome and stained with Reynolds lead citrate. Cell cross-section was imaged with a JEOL 100CX TEM.

3.2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Plasmids containing a partial 16S rRNA gene fragment from strain BL-DC-8 were constructed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Inserts were produced by performing PCR using universal bacterial primers 27f and 1392r. Following growth of recombinant *E. coli* cells in Luria-Bertani medium (1.0% tryptone, 0.5% yeast extract, 1.0%

NaCl, pH 7.0) amended with 50 µg/L kanamycin, plasmid DNA was extracted and purified using an UltraClean Mini Plasmid Prep kit (MoBio Laboratories, Carlsbad, CA). Plasmid DNA containing a “Dehalococcoides” strain 16S rRNA gene fragment insert was obtained by using the same method as described above but using DNA from BL-DHC-4, a putative “Dehalococcoides” strain pure culture (described in chapter 2) as the starting material.

Biomass was collected by centrifuging 15 mL culture at 8,200×g for 40 min at 4 °C. Total bacterial DNA was extracted from cell pellet by using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories, Carlsbad, CA). 16S rRNA genes from the samples and two plasmids were amplified using universal bacterial primers 341f (which included a 40 bp GC-clamp attached to the 5’end) and 907r as described previously (Li and Moe, 2004). Deionized water was used as template for negative control. Sequence of the 341f primer was 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCA G-3', and sequence of the 907r primer was 5'-CCGTCAATTCMTTTRAGTTT-3' (where M=C:A, R=A:G).

The PCR products were purified with the UltraClean PCR Purification kit and electrophoresed in a 30-mL polyacrylamide DGGE gel containing a denaturing gradient ranging from 40% to 70% denaturant (where 100% denaturant contains 7 M urea and 40% (v/v) formamide) cast using a BioRad Model 475 Gradient Delivery System. 8 µL purified PCR product mixed with 8 µL 2×loading dye was loaded into individual lanes of the gel. DGGE was performed using a D-Code Universal Mutation Detection System (BioRad Laboratories, Hercules, CA) in 1×TAE buffer at 60 °C and 65 V for 16 h. The gel was stained in ethidium bromide (1 µg/mL) solution for 20 min, and destained in deionized water for 20 min prior to imaging by using the ChemiDoc XRS gel documentation system (BioRad, Laboratories, Hercules, CA).

3.2.5 Southern Blotting

Southern blotting (VacuGene XL blotting system, Amersham Biosciences) was performed by Brain Rash using genomic DNA digested with PvuII, BamHI and TaqI restriction endonucleases in conjunction with a digoxigenin-labelled universal bacterial 16S rRNA gene probe (generated using PCR products of universal bacterial primers 357f and 519r [Rainey *et al.*, 1996] with *Escherichia coli* DNA as template) and gene probes specifically targeting 16S rRNA gene sequences unique to strains BL-DC-8 and BL-DC-9 (generated using PCR products of primers BL-DC-631F and BL-DC-796R [see below] with strain BL-DC-8 DNA as template). DNA digest products were separated on a 0.5% agarose gel. DNA was transferred to nylon membranes using the alkaline transfer method using the VacuGene XL blotting system (Amersham Biosciences) according to the manufacturer's instructions. Membranes were hybridized with probe according to manufacturer's instructions, and results were visualized using the eagle eye.

3.2.6 16S rRNA Gene Sequencing and Phylogenetic Analysis

Biomass from 15 mL culture was harvested by centrifugation at 8,200×g for 40 min at 4°C. Supernatant was decanted, and genomic DNA was extracted from pelleted cells using the UltraClean Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA). Extracted DNA was eluted in sterile water instead of Tris buffer. 16S rRNA gene sequences were determined as described previously (Rainey *et al.*, 1996) using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Foster City, CA) and an ABI 377 Automated DNA Sequencer.

Sequences were manually verified using BioEdit version 4.7.8 (Hall, 1999). The 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9 were aligned against previously determined sequences available from the public databases. Phylogenetic analyses were

performed by Fred A. Rainey using ARB (Ludwig *et al.*, 2004). The neighbor joining algorithm was used to build the phylogenetic tree, with Jukes-Cantor correction (Jukes and Cantor, 1969) followed by bootstrap analysis with Phylip 3.62 (Felsenstein, 2004).

3.2.7 Dechlorination Activities

The ability of strains BL-DC-8 and BL-DC-9 to dechlorinate a variety of chloroethenes, chloroalkanes, and chlorobenzenes was evaluated in triplicate culture bottles containing 15 mL basal medium. 10% H₂ gas mixture was provide in the headspace and chlorinated compounds were amended to reach approximately 0.5 mM in the aqueous phase after equilibration. Each bottle received a 2% or 3% (v/v) inoculum of BL-DC-8 or BL-DC-9 previously grown with 1,1,2-TCA. Negative controls were constructed exactly the same except that they did not receive any inoculum. Additional controls were prepared by inoculating serum bottles with strains BL-DC-8 and BL-DC-9 but without addition of chlorinated compound. Triplicate bottles were sacrificed at each time interval for analysis of parent compounds, degradation products, and 16S rRNA gene copy numbers.

3.2.8 Growth, Biochemical Characteristics

Growth temperature for the isolates was determined by testing at 8 °C, 15 °C, 18 °C, 20 °C, 23.5 °C, 28 °C, 30 °C, 34 °C, 37 °C, and 45 °C. Growth of the isolates was determined at pH 5.0, 5.5, 6.0, 6.5, and 7.0 using 20 mM MES buffer and 7.0, 7.5, 8.0, 8.5, and 9.0 with 20 mM Tris buffer. Experiments were conducted in 25 mL serum bottles containing 15 mL basal medium amended with 0.5 mM 1,2-DCP as electron acceptor and gas headspace comprised of H₂/CO₂/N₂ gas mixture (10:10:80, v/v/v) Six bottles were set up for each tested temperature or pH and received 3% (v/v) inoculum from an active 1,2-DCP dehalogenating BL-DC-8 or BL-DC-9 culture which was purged in advance to avoid transferring propene. Samples from all bottles

were analyzed at the end of 1 or 2 weeks and 2 months using GC-FID. Bottles were scored as positive for dehalogenation if >3% of the total amended 1,2-DCP was dechlorinated.

The ability of the isolates to use different electron donors was tested in triplicate in 68 mL serum bottles containing 40 mL anaerobic basal medium. Potential electron donors, supplied from filter-sterilized 100× or 10× stock solutions, were acetate (5 mM), butyrate (5 mM), citrate (5 mM), fumarate (5 mM), lactate (5 mM), malate (5 mM), propionate (5 mM), pyruvate (5 mM), succinate (5 mM), ethanol (5 mM), methanol (5 mM), fructose (5 mM), glucose (5 mM), lactose (5 mM), yeast extract (0.5 g/L) and methyl ethyl ketone (MEK) (5 mM). Each prepared bottle received 3% inoculum from an active 1,2-DCP dehalogenating BL-DC-8 or BL-DC-9 culture which was purged with CO₂/N₂ (5:95, v/v) gas mixture in advance to avoid transferring H₂ and propene. Neat 1,2-DCP was added to each bottle to reach a final aqueous-phase concentration of approximately 0.5 mM. All bottles were incubated for two months prior to analysis using GC-FID. Additional positive control bottles were set up with acetate as a carbon source and 10% H₂ (plus 10% CO₂ and 80% N₂) in the headspace as electron donor.

The ability of the isolates to utilize inorganic electron acceptors nitrate (5 mM), sulfate (5 mM), sulfite (5 mM) and oxygen (2% in headspace) were tested in 25 mL serum bottles containing 15 mL basal medium without any chlorinated compounds. H₂/CO₂/N₂ gas mixture (10:10:80, v/v/v) was provided in the headspace. Reducing reagents were omitted from the bottles in which oxygen was tested as electron acceptor. Each inorganic electron acceptor was tested in six bottles and received 3% (v/v) inoculum from an active 1,2-DCP dehalogenating BL-DC-8 or BL-DC-9 culture. All bottles were incubated for two months prior to analysis via ion chromatography (IC).

3.2.9 Oxygen and Salt Tolerance Test

To determine ability of the isolates to grow in the presence of O₂, 25 mL serum bottles

each containing 15 mL anaerobic basal medium were inoculated with BL-DC-8 or BL-DC-9 culture, and then headspace was purged with filter-sterilized oxygen at a flow rate of 0.5 L/min for 5 min. 1,2-DCP was spiked into each bottle to reach approximately 0.5 mM in the aqueous phase, and then 1.5 mL filter-sterilized H₂ was injected into the gas headspace as the electron donor. Bottles remained aerobic, as indicated by resazurin, during the incubation. Dehalogenation activities were analyzed periodically using GC-FID.

To determine ability of the strains to reductively dechlorinate following short-term O₂ exposure, serum bottles were prepared as described above, but then re-purged with filter-sterilized H₂/CO₂/N₂ gas mixture after purging with O₂ but before addition of 1,2-DCP. The medium became anaerobic within a few hours as indicated by resazurin color change. Triplicate bottles were sacrificed periodically to determine dehalogenation activities using GC-FID.

The ability of the isolates to grow at various NaCl concentrations was tested in the range of 0.1 (background level in growth medium), 1%, 2% and 3% (m/v) using 25 mL serum bottles containing 15 mL basal medium amended with 0.5 mM 1,2-DCP. Triplicate bottles were sacrificed to assess dehalogenation activities using GC-FID.

3.2.10 Pulsed Field Gel Electrophoresis (PFGE)

Genomic DNA was prepared from 40 mL BL-DC-8 and BL-DC-9 cultures according to the instruction of the CHEF Bacterial Genomic DNA Plug Kit (BioRad Laboratories Inc., Hercules, CA). Samples were electrophoresed at 14 °C through 1% (w/v) agarose gel in 1×Tris-acetate-EDTA buffer (BioRad, Laboratories, Hercules, CA) at 6 V/cm for 26 hrs with a CHEF-DR III Pulsed Field Electrophoresis System (BioRad Laboratories Inc., Hercules, CA). The separation angle was 120 ° and the switch time was set from 60 to 120 seconds. The gel was stained for 40 min in distilled water containing 0.5 µg/ mL ethidium bromides after the electrophoresis. Then gel was destained with distilled water for 40 min prior to imaging using a

ChemiDoc XRS gel documentation system (BioRad Laboratories Inc., Hercules, CA). DNA sizes were estimated by comparing band positions to N0345S yeast chromosome PFG markers (New England Biolabs Inc., Ipswich, MA).

3.2.11 G+C Content of DNA

Cells of strains BL-DC-8 and BL-DC-9 were collected from 2.4 L cultures of each strain by centrifuging at 8,200×g for 40 min at 4 °C. DNA was isolated using an UltraClean Microbial DNA Isolation kit (MoBio Laboratories Inc., CA). Genomic G+C content was determined by HPLC as described by Mesbah *et al.* (1989) in the laboratory of Milton S. da Costa (Universidade de Coimbra, Coimbra, Portugal).

3.2.12 qPCR

Details regarding development and testing of the quantitative real-time PCR (qPCR) protocol for determining 16S rRNA gene copy numbers as a function of time during dechlorination experiments is presented in Chapter 5 of this dissertation. Briefly, plasmid DNA containing a partial 16S rRNA gene insert from strain BL-DC-8 was used as standard to quantify the 16S rRNA gene concentrations. Plasmid concentrations were measured using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories Inc., Hercules, CA) at 260 nm. A conversion factor of 50 µg/mL double strand DNA corresponding to an absorbance of 1.0 at 260 nm was applied in the calculation. Serial dilutions of DNA standards were made with a factor of 10 and the threshold cycle values at each dilution level were obtained in the qPCR reactions. Calibration curves were made by plotting the log-transformed gene copy number against the threshold cycle values. The 16S rRNA gene copy number in a qPCR reaction was calculated as described by Ritalahti *et al.* (2006) in equation 1.

$$\text{gene copies/reaction} = (2 \mu\text{L/reaction}) (\text{DNA concentration}[\text{ng}/\mu\text{L}]) \left(\frac{1 \text{ g}}{1,000^3 \text{ ng}} \right) \left(\frac{1 \text{ mol bp DNA}}{660 \text{ g DNA}} \right)$$

$$\times \left(\frac{6.023 \times 10^{23} \text{ bp}}{\text{mol bp}} \right) \left(\frac{1 \text{ copy}}{\text{genome or plasmid size [bp]}} \right) \quad (1)$$

The primer set BL-DC-631f and BL-DC-796r was designed to specifically target the 16S rRNA gene of the isolates (see Chapter 5 for details). qPCR was performed with a iCycler iQ PCR thermal cycler detection system (Bio-Rad Laboratories Inc., Hercules, CA) and a qPCR supermix kit (SYBR GreenER, Invitrogen Inc., Carlsbad, CA). Each qPCR reaction (25 μ L) contained: 12.5 μ L 2 \times supermix, 500 nM each primer, 2 μ L DNA extract, 10 μ L DNA free water. PCR tubes were sealed with optically clear cap strips and were placed on ice to maintain enzyme activities before reaction. PCR thermal protocol was 50°C for 2 min and 95°C for 8.5 min, followed by 40 cycles of 95°C for 15 sec and annealing for 1 min.

3.2.13 Analytical Methods

Quantitative analysis of chlorinated compounds, ethane, and propene were performed with an HP model 6890 gas chromatograph equipped with a flame ionization detector (FID) and GS-GasPro capillary column (60 m \times 0.32 mm I.D., J&W P/N 113-4362) as described in Chapter 2. Nitrate, and sulfate were measured using an ion chromatograph (Dionex, Sunnyvale, CA) equipped with an AG14a guard column, AS14a analytical column, and conductivity detector. Samples were introduced via a 25 μ L injection loop. Isocratic elution was performed at a flow rate of 1.0 mL/minute and temperature of 30°C.

3.3 Results

3.3.1 Morphology and Culture Purity

Strains BL-DC-8 and BL-DC-9 were small, gram-negative anaerobic bacteria when they were grown in the 1,1,2-TCA amended basal medium. Spore or flagella were not observed using phase contrast microscopy. SEM and TEM microscopic examinations revealed homogenous

morphology in cultures of both BL-DC8 and BL-DC-9 and a cell diameter of 0.3 μm to 0.6 μm for both (Fig. 3.1).

Several approaches were adopted to investigate the culture purity of the two isolates in addition to microscopic observations. (1) Genomic DNA was extracted from both cultures and amplified with universal bacterial primer set (341f and 907r) to yield a segment approximately 580 bp in length. Only single bands were observed in denaturing gradient gel electrophoresis (DGGE), and the band positions for strains BL-DC-8 and BL-DC-9 were substantially different from that from putative “Dehalococcoides” strain DHC-4 (Fig. 3.2). (2) Single peaks were observed in melting curve analysis following qPCR amplification using primers specific to BL-DC-8 and BL-DC-9 (Fig. 3.3). (3) In Southern blotting, strains BL-DC-8 and BL-DC-9 both generated identical patterns with a single band using both universal or specific 16S rRNA gene probes (data not shown).

3.3.2 Phylogenetic Analysis

Strains BL-DC-8 and BL-DC-9 shared nearly identical 16S rRNA gene sequences (one base difference, 99.93% identity). Phylogenetic comparisons with members of the domain Bacteria showed that BL-DC-8 and BL-DC-9 represent a distinct lineage within the phylum *Chloroflexi* (Fig. 3.4). Nearly complete 16S rRNA gene sequences comprising 1455 nucleotide positions determined for strains BL-DC-8 and BL-DC-9 were deposited in GenBank with accession numbers EU679418 and EU679419, respectively.

An uncultured bacterial clone Er-MS-91 (unpublished, gene bank accession number EU542454), derived from PCBs dechlorinating sludge, shared 94% sequence identities to both strains. Their closest cultured phylogenetic relatives are “Dehalococcoides” strains with 90% sequence identities.

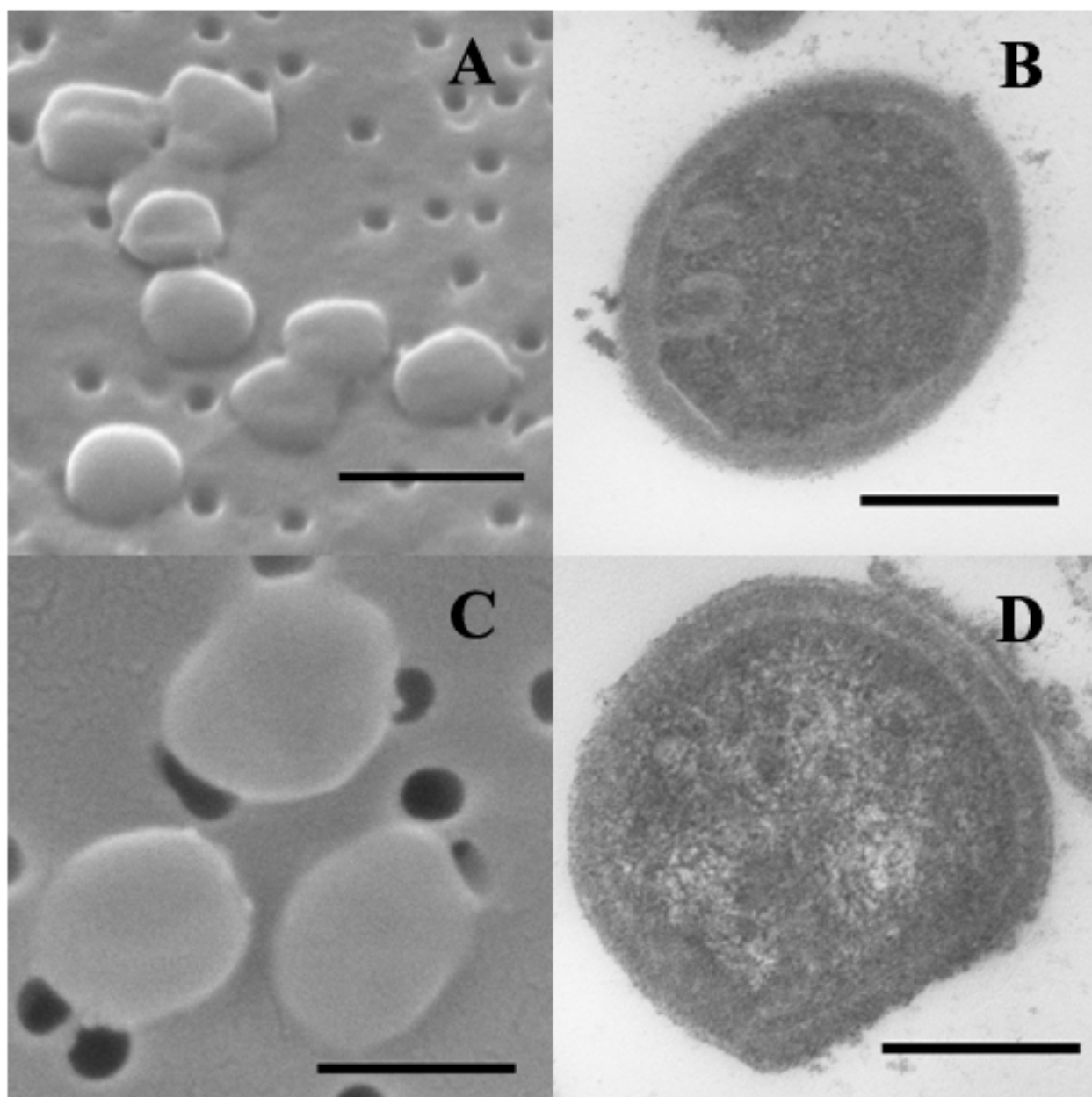


Figure 3.1. SEM and TEM images of strains BL-DC-8 and BL-DC-9. (A) SEM image of BL-DC-8. Scale bar, 1 μm . (B) TEM image of BL-DC-8. Scale bar, 0.2 μm . (C) SEM image of BL-DC-9. Scale bar, 0.5 μm . (D) TEM image of BL-DC-9. Scale bar, 0.2 μm .

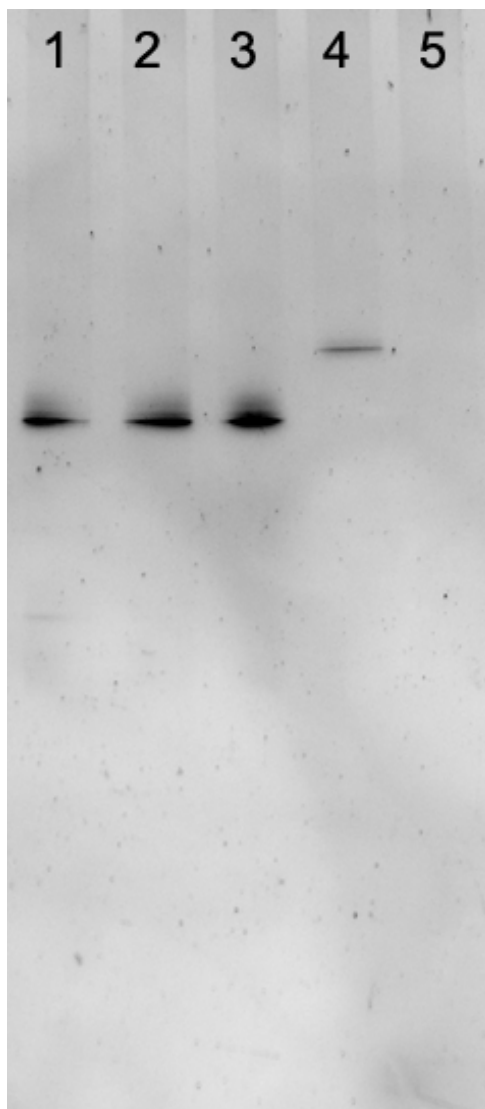


Figure 3.2. Negative image of DGGE fingerprints of PCR products from BL-DC-8 (lane 1) and BL-DC-9 (lane 2) genomic DNA extracts. Lane 3 is the PCR products from BL-DC-8 plasmid and lane 4 is the PCR product from plasmid containing partial “Dehalococcoides” strain 16S rRNA gene. Lane 5 is DGGE-PCR negative control with DNA-free water as template in DGGE-PCR.

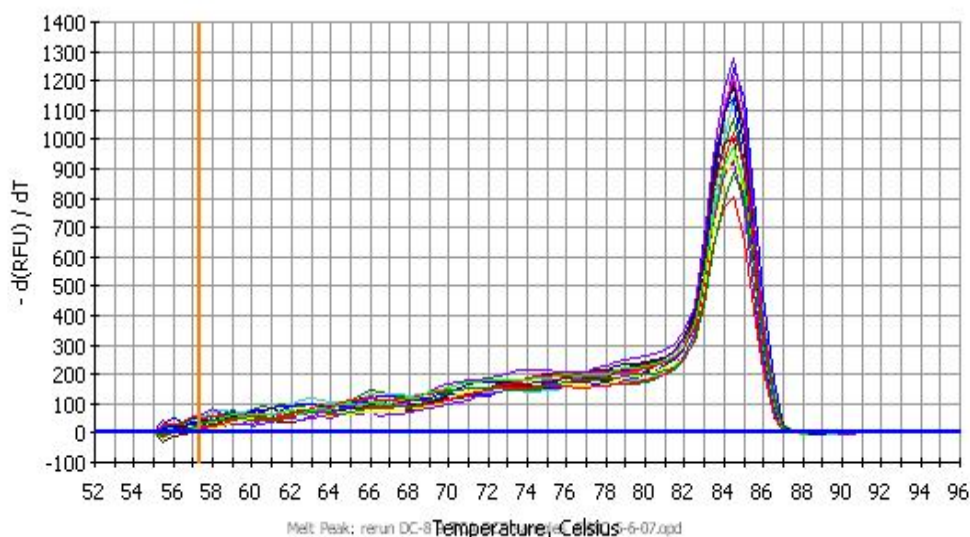


Figure 3.3. qPCR melting curves of the 16S rRNA gene segments of strains BL-DC-8 and BL-DC-9 amplified using BL-DC-631f and BL-DC-796r primer set.

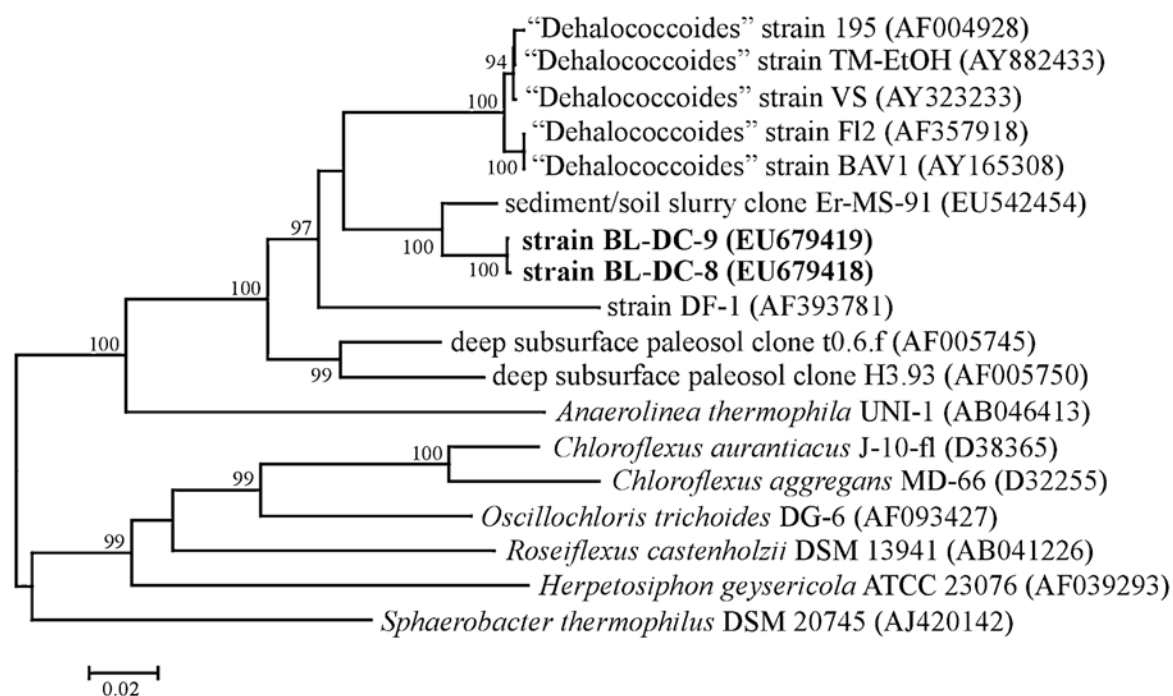


Figure 3.4. Phylogenetic relationship of strains BL-DC-8 and BL-DC-9 to species in the phylum *Chloroflexi* (Hugenholtz and Stackebrandt, 2004) based on 16S rRNA gene sequences. Bootstrap values expressed as a percentage of 1000 resamplings are shown at the branch points. For branch points where no value is shown, bootstrap values are less than 95%.

3.3.3 Dehalogenating Activities

The dehalogenation capacities of isolates BL-DC-8 and BL-DC-9 were investigated by testing a variety of chlorinated compounds including chlorinated alkenes, alkanes, and benzenes. In addition to 1,1,2-TCA, strains BL-DC-8 and BL-DC-9 also coupled microbial growth to reductive dehalogenation of several other vicinally chlorinated aliphatic alkanes (Fig. 3.5). 1,2-Dichloropropane (1,2-DCP) was transformed to propene, 1,2-dichloroethane (1,2-DCA) was transformed to ethene, and 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA) was transformed to a mixture of *cis*- and *trans*-1,2-dichloroethene (DCE) and trichloroethene (TCE). Transformation of 1,1,2,2-TeCA to TCE also occurred in sterile negative controls, however, consistent with an abiotic reaction pathway reported previously (Cooper *et al.*, 1987; Chen *et al.*, 1996). *Cis*- and *trans*-1,2-DCE were not detected in abiotic negative controls and may have been the sole metabolic products from 1,1,2,2-TeCA transformation by isolates BL-DC-8 and BL-DC-9.

Incubated under the testing conditions for 2 months at 30 °C, the isolates did not reductively dehalogenate alkanes containing only a single chlorine substituent (e.g., 1-chloropropane, 2-chloropropane), chlorinated alkenes (PCE, TCE, *cis*-DCE, *trans*-DCE, vinyl chloride), or chlorinated benzenes (monochlorobenzene and 1,2-dichlorobenzene). Dehalogenation-linked growth with 1,1,2,2-TeCA, 1,1,2-TCA, 1,2-DCA or 1,2-DCP was determined by investigating the increase of 16S rRNA gene copies during the dehalogenation using a qPCR approach. The increase in concentration of 16S rRNA gene copies during dehalogenation of 1,2-DCP, 1,2-DCA, 1,1,2-TCA and 1,1,2,2-TeCA were plotted against the increase dehalogenation daughter products for each time step to calculate cell yields (Fig. 3.6).

The net cell yields of these two strains were comparable, and close to those previously reported “Dehalococcoides” strain BAV1 when 1,1,2-TCA was supplied as the electron acceptor (He *et al.*, 2003b), strains BL-DC-8 and BL-DC-9 exhibited the highest yields at $0.18 \pm 0.01 \times 10^8$

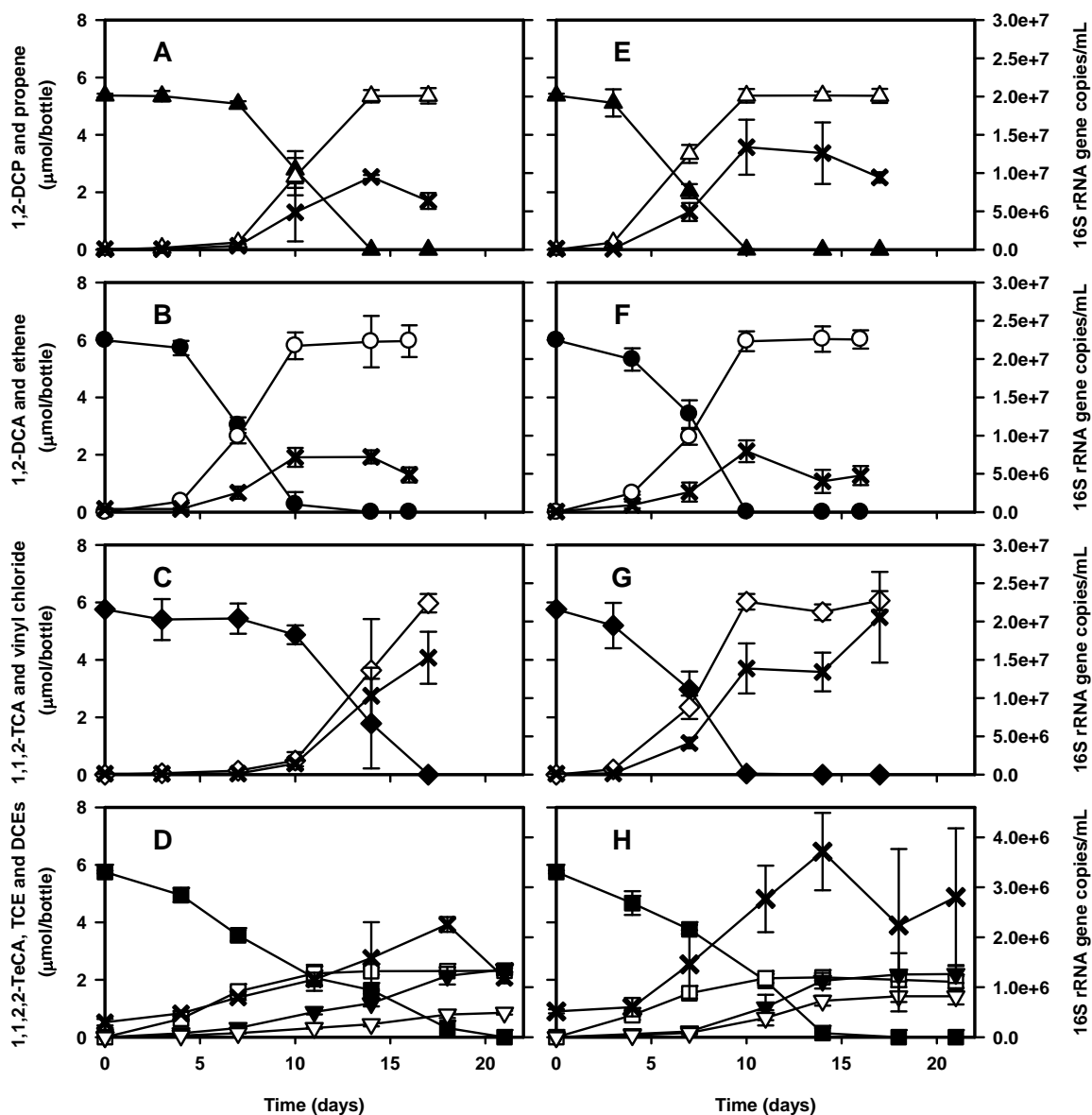


Figure 3.5. Reductive dechlorination of 1,2-DCP (A, E), 1,2-DCA (B, F), 1,1,2-TCA (C, G) and 1,1,2,2-TeCA (D, H) by strains BL-DC-9 (A, B, C, D) and BL-DC-8 (E, F, G, H). (▲) 1,2-DCP; (Δ) Propene; (●) 1,2-DCA; (○) Ethene; (◆) 1,1,2-TCA; (◇) Vinyl chloride; (■) 1,1,2,2-TeCA; (□) TCE; (▼) *cis*-1,2-DCE; (▽) *trans*-1,2-DCE; (×) 16S rRNA gene copies. Error bars represent standard deviation of triplicate bottles. Scale bars on the left and right apply to both graphs in each row.

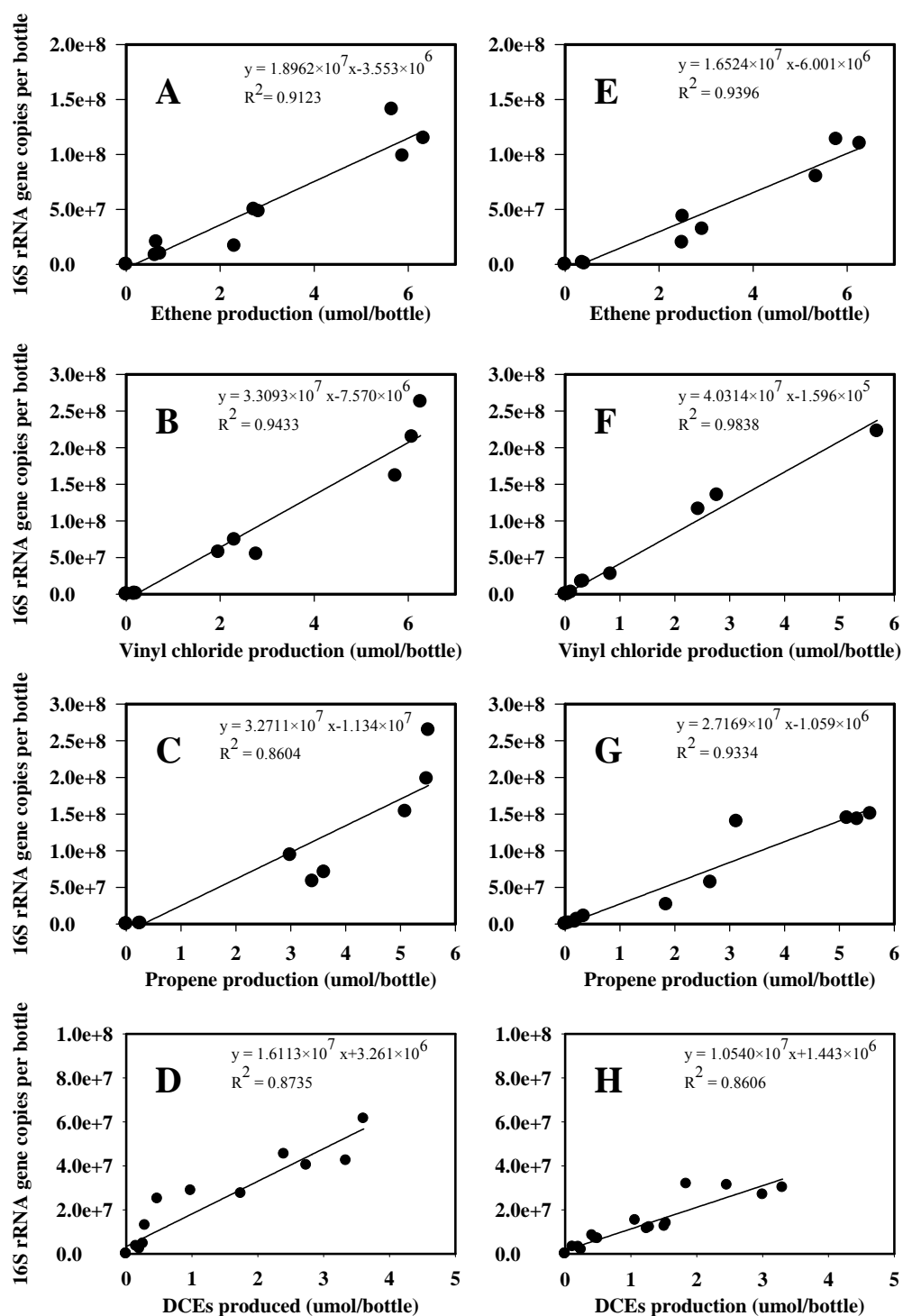


Figure 3.6. Yields of 16S rRNA genes of strains BL-DC-8 (A,B,C,D) and BL-DC-9 (E,F,G,H) linked with the dehalogenations of 1,2-DCA (A,B), 1,1,2-TCA (C,D), 1,2-DCP (E,F) and 1,1,2,2-TeCA (G,H).

Table 3.1. Summary of dehalogenation linked growth yields of “Dehalococcoides” species and strains BL-DC-8 and BL-DC-9

Strain	Dehalogenation	Yield ^a	Reference
“Dehalococcoides” strain FL2	PCE+TCE→vinyl chloride	$0.73 \pm 0.02 \times 10^8$	He <i>et al.</i> , 2005
	TCE→vinyl chloride	$0.78 \pm 0.09 \times 10^8$	
	<i>Cis</i> -DCE→vinyl chloride	$0.84 \pm 0.08 \times 10^8$	
	<i>Trans</i> -DCE→vinyl chloride	$0.81 \pm 0.15 \times 10^8$	
“Dehalococcoides” strain BAV1	<i>Cis</i> -DCE→ethene	0.58×10^8	He <i>et al.</i> , 2003b
	Vinyl chloride→ethene	0.62×10^8	
“Dehalococcoides” strain VS	Vinyl chloride→ethene	5.9×10^8	Cupples <i>et al.</i> , 2003
“Dehalococcoides”	1,2-DCA→ethene	$1.6 \pm 0.8 \times 10^8$	Duhamel and Edwards, 2007
	Vinyl chloride→ethene	$1.5 \pm 0.3 \times 10^8$	
	<i>Cis</i> -DCE →ethene	$0.9 \pm 0.3 \times 10^8$	
Strain BL-DC-8	1,2-DCA→ethene	$0.09 \pm 0.01 \times 10^8$	This study
	1,1,2-TCA→vinyl chloride	$0.18 \pm 0.01 \times 10^8$	
	1,1,2,2-TeCA→DCEs	$0.08 \pm 0.02 \times 10^8$	
	1,2-DCP→propene	$0.18 \pm 0.02 \times 10^8$	
Strain BL-DC-9	1,2-DCA→ethene	$0.09 \pm 0.01 \times 10^8$	This study
	1,1,2-TCA→vinyl chloride	$0.20 \pm 0.01 \times 10^8$	
	1,1,2,2-TeCA→DCEs	$0.06 \pm 0.01 \times 10^8$	
	1,2-DCP→propene	$0.14 \pm 0.01 \times 10^8$	

^a: Unit of growth yield is normalized as 16S rRNA gene copies/ $\mu\text{mol Cl}^-$ released.

and $0.20 \pm 0.01 \times 10^8$ 16S rRNA gene copies/ $\mu\text{mol Cl}^-$ released (Table 3.1).

3.3.4 Growth and Biochemical Characteristics

Although both strains were routinely grown with ampicillin (1g/L) and vancomycin (100 mg/L) in the growth media, they could be successively transferred to media lacking antibiotics with no apparent adverse impact on the rate or extent of dechlorination. Both strains could not be cultured on agar or gellan gum solidified medium.

Growth was observed for strains BL-DC-8 and BL-DC-9 only when a vicinally chlorinated alkane was provided as an electron acceptor including 1,1,2,2-TeCA, 1,1,2-TCA, 1,2-DCA, and 1,2-DCP, and H_2 was provide as electron donor. NO_3^- , SO_4^{2-} or O_2 could not be utilized as inorganic electron acceptor. H_2 could not be replaced by acetate, butyrate, citrate, fumarate, lactate, malate, propionate, pyruvate, succinate, ethanol, methanol, fructose, glucose, lactose, yeast extract, or methyl ethyl ketone.

The temperature range for dechlorination was 20 °C to 34 °C for strain BL-DC-8 and 20 °C to 37 °C for strain BL-DC-9; optimum was 28 °C to 34 °C for both (Fig. 3.7). The pH range for dechlorination was 6.0 to 8.0 with optimum 7.0 to 7.5 for both strains (Fig. 3.8). No growth occurred at pH less than 5.5 or greater than 8.5. Both strains dechlorinated 1,2-DCP at NaCl concentrations up to 2% (m/v) but not 3%. No dechlorination occurred in the presence of oxygen, but dechlorination activity could be completely recovered after prolonged incubation following brief (5 min) oxygen exposure.

3.3.5 Genome Size and DNA mol % G+C Content

The genome sizes of both strains were estimated to be approximate 2 Mbp using PFGE (Fig. 3.9). Genomic DNA G+C content were determined to be 53.95 and 53.8 mol% for strains BL-DC-8 and BL-DC-9, respectively.

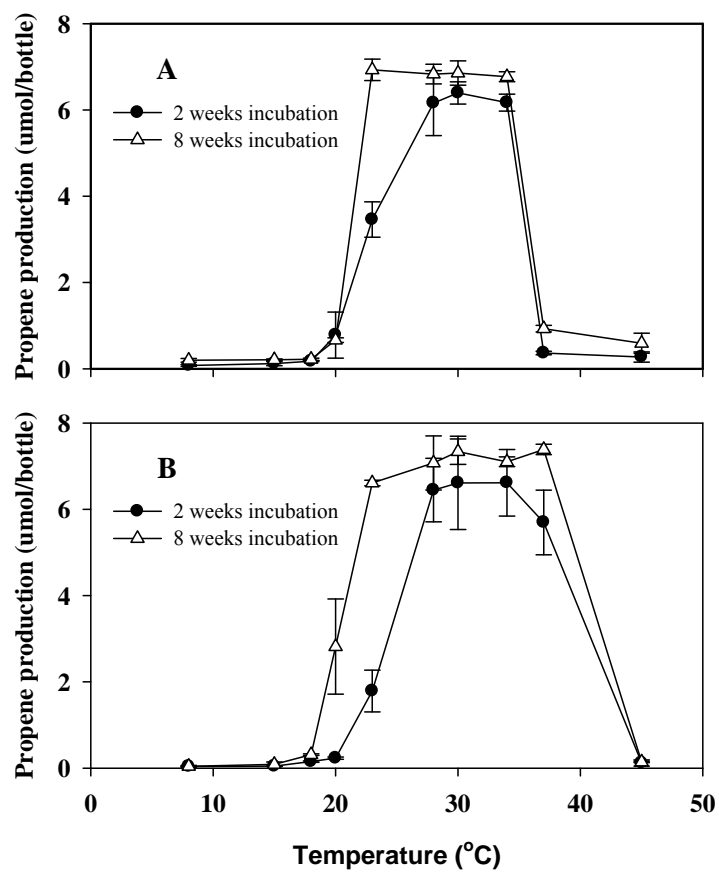


Figure 3.7. Growth temperature range for stains BL-DC-8 (A) and BL-DC-9 (B) indicated by the dehalogenation of 1,2-DCP.

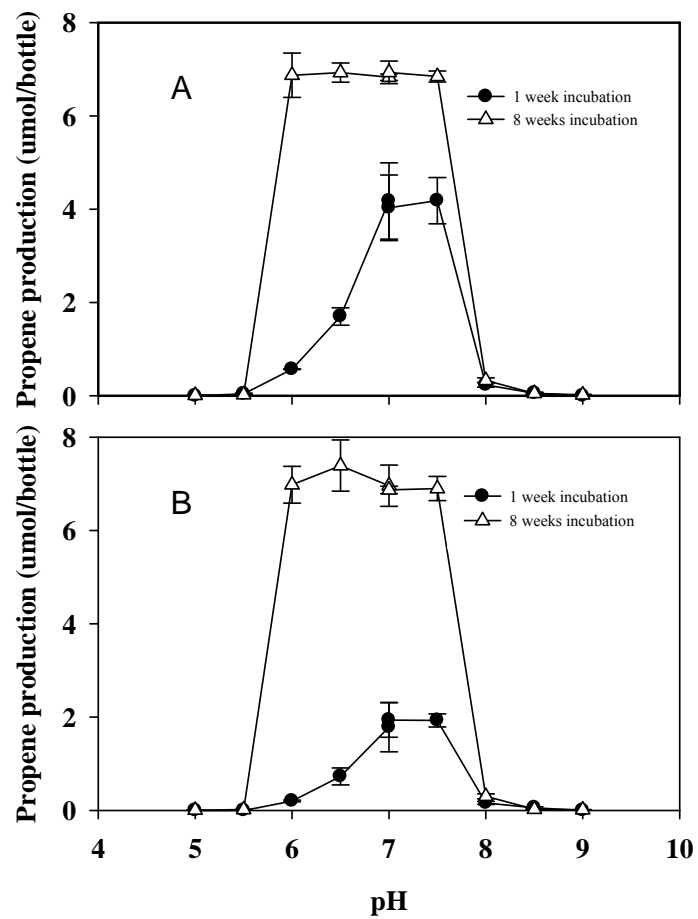


Figure 3.8. Growth pH range for stains BL-DC-8 (A) and BL-DC-9 (B) indicated by the dehalogenation of 1,2-DCP.

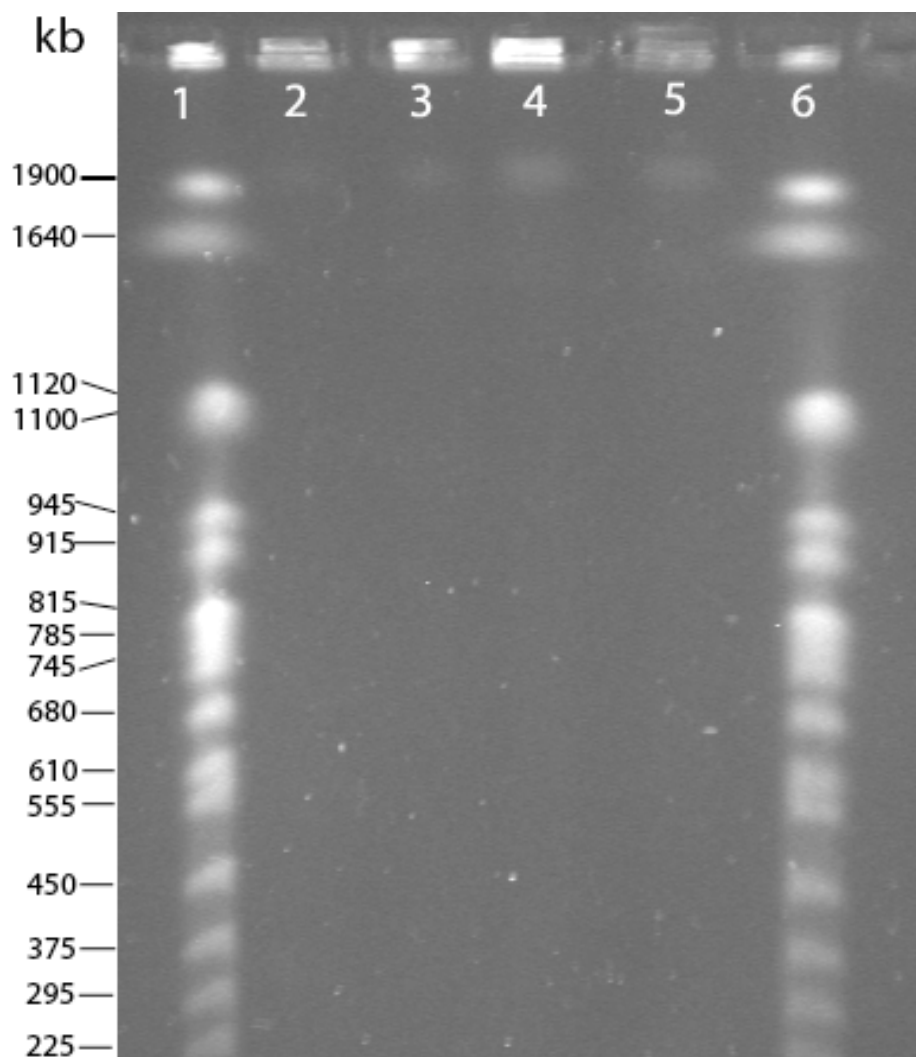


Figure 3.9. PFGE image of the genomic DNA from strains BL-DC-8 (lanes 2 and 3) and BL-DC-9 (lanes 4 and 5). Lane 1 and 6, yeast chromosome PFG marker N0345S (New England Biolabs, Ipswich, MA).

3.4 Discussion

Strains BL-DC-8 and BL-DC-9 were demonstrated to be pure cultures by microscopic observations, DGGE examination and southern blotting. Both strains exclusively dehalogenated vicinally chlorinated alkanes via dihaloelimination reactions in growth-linked processes. Exclusively dihaloelimination reactions had only been reported in the previously isolated *Desulfitobacterium dichloroeliminans* strain DCA1, which dihaloeliminated 1,1,2-TCA, 1,2-DCA and 1,2-DCP (De Wildeman *et al.*, 2003). Strain DCA1, however, was unable to dehalogenate 1,1,2,2-TeCA, and it is phylogenetically unrelated to strains BL-DC-8 and BL-DC-9, falling within the phylum *Firmicutes*. Several studies on reductively dechlorinating mixed cultures have also reported that 1,1,2-TCA, and 1,2-DCP were directly dehalogenated to vinyl chloride and propene, respectively, without any detectable intermediates (Löffler *et al.*, 1997; Ritalahti and Löffler, 2004; Schlotelburg *et al.*, 2000, 2002; Grostern and Edwards, 2006).

Besides *Desulfitobacterium dichloroeliminans* strain DCA1, dehalogenation of chlorinated alkanes has been linked previously to uncharacterized *Dehalobacter*, *Desulfuromonas* and “Dehalococcoides” strains in various studies (Löffler *et al.*, 1997; Ritalahti and Löffler, 2004; Schlotelburg *et al.*, 2000, 2002; USGS, 2003). Isolation of strains BL-DC-8 and BL-DC-9, which are not closely related to any of these previously described bacterial populations, suggests that dehalogenating populations may be much more diverse than previously known. Further studies on dihaloelimination and the organisms that can carry out such reactions may lead to a more comprehensive understanding of the ecology, phylogeny and physiology of dehalogenating populations, and thus contribute to development of cost-effective methods restore hazardous waste sites.

Phylogenetically, strains BL-DC-8 and BL-DC-9 fall within the phylum *Chloroflexi*. Their closest cultured relatives are the “Dehalococcoides” strains, but with 16S rRNA gene

sequence similarities of only 90%, strains BL-DC-8 and BL-DC-9 clearly represent a new genus. Previously described “Dehalococcoides” strains are capable of utilizing all known chlorinated ethenes, some chlorinated benzenes, and the chloroalkane 1,2-DCA in growth linked processes (Maymo-Gatell *et al.*, 1997; Adrian *et al.*, 2000; He *et al.*, 2003b; Cupples *et al.*, 2003; Fennell *et al.*, 2004; He *et al.*, 2005). The spectrum of chlorinated substrates utilized, however, varies substantially between the various “Dehalococcoides” strains.

Similar to what has been described previously for “Dehalococcoides” strains, strains BL-DC-8 and BL-DC-9 appeared to grow only when halogenated compounds were provided as electron acceptors and H₂ was provided as an electron donor. Unlike the “Dehalococcoides” strains described previously, strains BL-DC-8 and BL-DC-9 appear to exclusively dechlorinate vicinally halogenated alkanes. Strains BL-DC-8 and BL-DC-9 were unable to utilize chlorinated ethenes or chlorinated benzenes. Since initially reported in 1995, “Dehalococcoides” strains have been found widely distributed at chloroethene-contaminated sites around the world and even some pristine environments (Hendrickson *et al.*, 2002; He *et al.*, 2005). Further research is needed to determine whether the bacterial group represented by strains BL-DC-8 and BL-DC-9 are similarly widely distributed in the environment.

3.5 Conclusions

Two novel anaerobic bacteria isolated from a 1,1,2-TCA enriched microcosm were characterized using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences shows these two strains, BL-DC-8 and BL-DC-9, to be distantly related to previously described genera. Both strains were demonstrated to be capable of coupling cell growth with the dehalogenation of a variety of chlorinated alkanes via what appear to be exclusively dihaloelimination reactions. Strains BL-DC-8 and BL-DC-9 grew best near neutral pH and in the temperature range of 28 °C to 34 °C.

CHAPTER 4. MICROBIAL REDUCTIVE DEHALOGENATION OF 1,2,3-TRICHLOROPROPANE

4.1 Introduction

1,2,3-Trichloropropane (1,2,3-TCP) is a chlorinated hydrocarbon used as an intermediate in chemical syntheses, as a solvent, and as an extractive agent (ATSDR, 1995). There are no known naturally occurring sources of the compound (WHO, 2003). It is produced in large quantities as a by-product during manufacture of epichlorohydrin (Bosma *et al.*, 2002; WHO, 2003). Historically, it has been present as an impurity in soil fumigants and nematocides (Zebarth *et al.*, 1998). A variety of animal studies indicate that 1,2,3-TCP is toxic and likely carcinogenic (ATSDR, 1992; WHO, 2003). 1,2,3-TCP is present as a contaminant in at least 20 of the National Priorities List sites identified by the US Environmental Protection Agency (ATSDR, 1995). It has also been detected in surface waters in various countries around the world including Japan, the Netherlands, and Slovakia (WHO, 2003). In 2001, the California Department of Health Services added 1,2,3-TCP to its list of unregulated chemicals for which monitoring was required in groundwater. Subsequently, 1,2,3-TCP was detected in over 200 public drinking water supply wells. Widespread recent detection in combination with a present lack of US federal regulations regarding maximum contaminant levels allowable in drinking water has led to it being regarded as an “emerging contaminant” (Book and Spath, 2007).

Microbial transformation of 1,2,3-TCP via oxidation with O_2 as an electron acceptor and via reduction with H_2 as an electron donor are both thermodynamically favorable (Dolfing and Janssen, 1994). Although aerobic cometabolic transformation of 1,2,3-TCP has been demonstrated previously (Bosma and Janssen, 1998), enzymes with weak catalytic activity toward 1,2,3-TCP oxidation have been identified (Banáš *et al.*, 2006; Monincová *et al.*, 2007), and a recombinant bacterial strain able to aerobically transform 1,2,3-TCP to a final product of

2,3-dichloro-1-propanol was reported (Bosma *et al.*, 2002), all attempts to isolate wild-type cultures able to utilize 1,2,3-TCP as a sole source of carbon or energy have failed (Bosma *et al.*, 2002; WHO, 2003). Additionally, implementation of aerobic in-situ bioremediation processes are hampered by limited ability to effectively transfer O₂ to the subsurface, and release of genetically modified bacteria in bioremediation remains controversial and subject to regulatory restrictions.

Anaerobic microbial processes have been widely used to achieve reductive dehalogenation of chlorinated ethenes in contaminated soils and groundwaters. Much less is known, however, about microbially mediated reductive dehalogenation of chlorinated alkanes in general and 1,2,3-TCP in particular. There have been only limited accounts of microbial reductive dechlorination of 1,2,3-TCP (Long *et al.*, 1993; Löffler *et al.*, 1997; Peijnenburg *et al.*, 1998; Hauck and Hegemann, 2000). Aside from the report that trace amounts of 1,2-dichloropropane and propene were detected after three months incubation of 1,2,3-TCP in a mixed culture able to transform 1,2-dichloropropane (Löffler *et al.*, 1997), dechlorination products have not been identified, and chloride release has not been documented. No microorganisms able to reductively dechlorinate 1,2,3-TCP have been described previously.

As described in Chapter 3, a variety of vicinally chlorinated alkanes were demonstrated to be reductively dehalogenated by the novel bacterial strains BL-DC-8 and BL-DC-9. The study described in this chapter was conducted to assess the abilities of these strains to reductively dehalogenate 1,2,3-TCP.

4.2 Materials and Methods

4.2.1 Chemicals

1,2,3-Trichloropropane (99%), allyl chloride (99%), allyl alcohol (99%), diallyl sulfide (97%), allyl mercaptan (technical grade, ~60%), and allyl methyl sulfide (98%) were purchased

from Sigma-Aldrich (St. Louis, MO). Diallyl disulfide (99.5%) was purchased from LKT Laboratories, Inc. (St. Paul, MN).

4.2.2 Medium Preparation and Culture Conditions

Experiments to assess potential of the strains to reductively dechlorinate 1,2,3-TCP were conducted using glass serum bottles (68 mL) fitted with Teflon-lined septa and aluminum crimp caps. Serum bottles were filled with 40 mL anaerobic basal medium with the same formulation used for isolation except that the chloride-containing salts NaCl, MgCl₂, NH₄Cl, and KCl were replaced with equal molar concentrations of corresponding bromide-containing salts. Gas headspace of each bottle was purged with H₂/CO₂/N₂ gas mixture. Replicate bottles were spiked with filter-sterilized 1,2,3-TCP (2 µL, ~19 µmol) to reach a final aqueous-phase concentration of approximately 0.5 mM. Each bottle received a 2% (v/v) inoculum of BL-DC-8 or BL-DC-9 previously grown with 1,1,2-trichloroethane. Negative controls were constructed exactly the same except that they did not receive any inoculum. Additional controls were prepared by inoculating serum bottles with strains BL-DC-8 and BL-DC-9 but without addition of 1,2,3-TCP. All bottles were inverted and statically incubated at 30 °C in dark. Triplicate bottles were sacrificed at regular time intervals for analysis of chlorinated solvents, degradation products, chloride, and 16S rRNA gene copy numbers.

4.2.3 DNA Extraction and qPCR

Biomass was collected at regular time intervals during the 1,2,3-TCP dehalogenation experiments. Bacterial genomic DNA was extracted and 16S rRNA gene quantification was performed according to the methods described in Chapter 3. Maximum specific growth rates were estimated by fitting the Gompertz model (Zweitering *et al.*, 1990) to 16S rRNA gene copy data using SigmaPlot 10.0.

4.2.4 Analytical Methods

1,2,3-TCP and its degradation products were measured using an HP model 6890 gas chromatograph (GC) equipped with a flame ionization detector and a DB-624 capillary column (60m×0.32mm I.D., Agilent 19091V-416). The oven temperature program was 40 °C for 5 min, increased at 20 °C/min to 260 °C and held for 3 min. Helium was used as carrier gas with a flow of 3.0 ml/min. Headspace gas samples collected in 100 µL gas-tight glass syringes (Hamilton, Baton Rouge, LA) were introduced to the GC via direct, splitless injection. Aqueous samples were introduced to the GC via a Tekmar 2016/3000 purge and trap autosampler and concentrator. Identification of volatile allyl-compound transformation products was confirmed using m/z spectra obtained using an HP model 5890 GC equipped with a mass selective detector (Agilent 5971), a DB-624 capillary column (30 m×0.25 mm×1.4 µm), and a Tekmar 2016 purge and trap concentrator.

Chloride was measured using an ion chromatograph as described in Chapter 3. S-allylcysteine was analyzed by C18-HPLC upon elution with 0.05 M KH₂PO₄ (pH 4.5)/methanol (97.5:2.5) at 220 nm (Lawson and Wang, 2005). S-allylcysteine analysis was performed by Silliker, Inc. (Orem, UT).

4.3 Results

4.3.1 Reductive Dehalogenation of 1,2,3-TCP

Tests to determine the ability of the strains to dechlorinate 1,2,3-TCP were conducted in low-chloride anaerobic basal medium supplemented with acetate and provided H₂ via gas headspace. Disappearance of 1,2,3-TCP was accompanied by stoichiometric release of inorganic chloride ions as well as microbial growth, measured by quantitative real-time PCR (qPCR) with a primer set targeting the 16S rRNA gene (Fig. 4.1). Maximum specific growth rates when growing with 1,2,3-TCP as an electron acceptor were estimated to be 0.17 and 0.15 days⁻¹,

corresponding to cell doubling times of 4.1 and 4.8 days, for strains BL-DC-9 and BL-DC-8, respectively. Dechlorination activity was maintained during repeated transfers of the strains in medium containing 1,2,3-TCP. No microbial growth, increase in chloride concentration, or degradation products were detected in negative controls.

For both strains, allyl chloride was the first dechlorination product observed, and it initially accumulated, reaching maximum concentrations of 1.34 ± 0.34 and 1.88 ± 0.52 μmol per bottle for strains BL-DC-8 and BL-DC-9, respectively. Allyl chloride was subsequently consumed by the end of the incubation period. Allyl alcohol, diallyl sulfide, and diallyl disulfide, appeared over time as products (Fig. 4.2). The identities of these allyl- compounds were confirmed by matching the retention times and mass spectra with standards. None of these compounds have been reported previously as products from microbial 1,2,3-TCP transformation. Production of less-chlorinated saturated alkanes (1,2-dichloropropane, 1-chloropropane, 2-chloropropane) and propene were not detected during 1,2,3-TCP dechlorination.

4.3.2 Abiotic Transformation of Allyl Chloride

In the overall pathway for 1,2,3-TCP dechlorination (Fig. 4.3), abiotic reactions involving the metabolite allyl chloride accounted for a portion of the chloride release. Allyl chloride is known to undergo hydrolysis in water with a short half-life, between 3.8 and 5.3 days at 30 °C, to form allyl alcohol and chloride (Krijgsheld and Gen *et al.*, 1986). Allyl chloride can also react with sulfides (present as a reducing agent in our growth medium), in aqueous solution to form diallyl-sulfides plus chloride (Xin-ke *et al.*, 2006). An experiment was conducted to investigate the abiotic behavior of ally chloride in the basal medium used to culture strains BL-DC-8 and BL-DC-9.

Experiments were conducted in uninoculated, sterile medium identical to the composition used in our study but amended with 1.2 mmol/L allyl chloride in aqueous phase. As shown in

Figure 4.4, abiotic degradation of allyl chloride took place. Abiotic transformation can be better fitted in an exponential decay model ($R^2=0.9940$) rather than a simple linear model ($R^2=0.8827$). The half-life of allyl chloride was found to be 2.0 days, with allyl alcohol, diallyl sulfide and diallyl disulfide as products.

4.3.3 Mass Balance

At the end of incubation of BL-DC-8 and BL-DC-9 cultures amended with 1,2,3-TCP, the products allyl chloride, allyl alcohol, diallyl sulfide, and diallyl disulfide accounted for 17% and 13% of carbon initially present in the form of 1,2,3-TCP. At least part of the gap in carbon balance can be attributed to further reactions involving products that were observed. For example, reaction of diallyl disulfide with cysteine (which was present as a reducing agent in our growth medium), is known to result in production of allyl mercaptan and S-allylmercaptocysteine (Lawson, 1996). Of these, allyl mercaptan was consistently detected in our study; however, it could not be accurately quantified due to the lack of a commercially available high-purity standard. Based on calibration curves prepared using technical grade allyl mercaptan (Sigma Aldrich, purity ~ 60%), maximum allyl mercaptan concentrations were estimated to be 0.38 ± 0.10 and 0.30 ± 0.21 $\mu\text{mol/bottle}$ for strains BL-DC-8 and BL-DC-9, respectively. S-allylcysteine, a known product of allyl halide reactions with cysteine (Maldonado *et al.*, 2003), was tentatively identified by HPLC as a product in our study, but at a concentration less than the method quantification limit of 12 μM . Trace levels of allyl methyl sulfide, a metabolite of allyl mercaptan in humans (Lawson and Wang, 2005), were detected via GC-MS.

4.4 Discussion

Production of allyl chloride during dehalogenation of 1,2,3-TCP is consistent with the previous report that these isolates coupled cell growth to reductive dihaloelimination of several other vicinally chlorinated aliphatic alkanes (Chapter 3). However, the enzymatic activity on

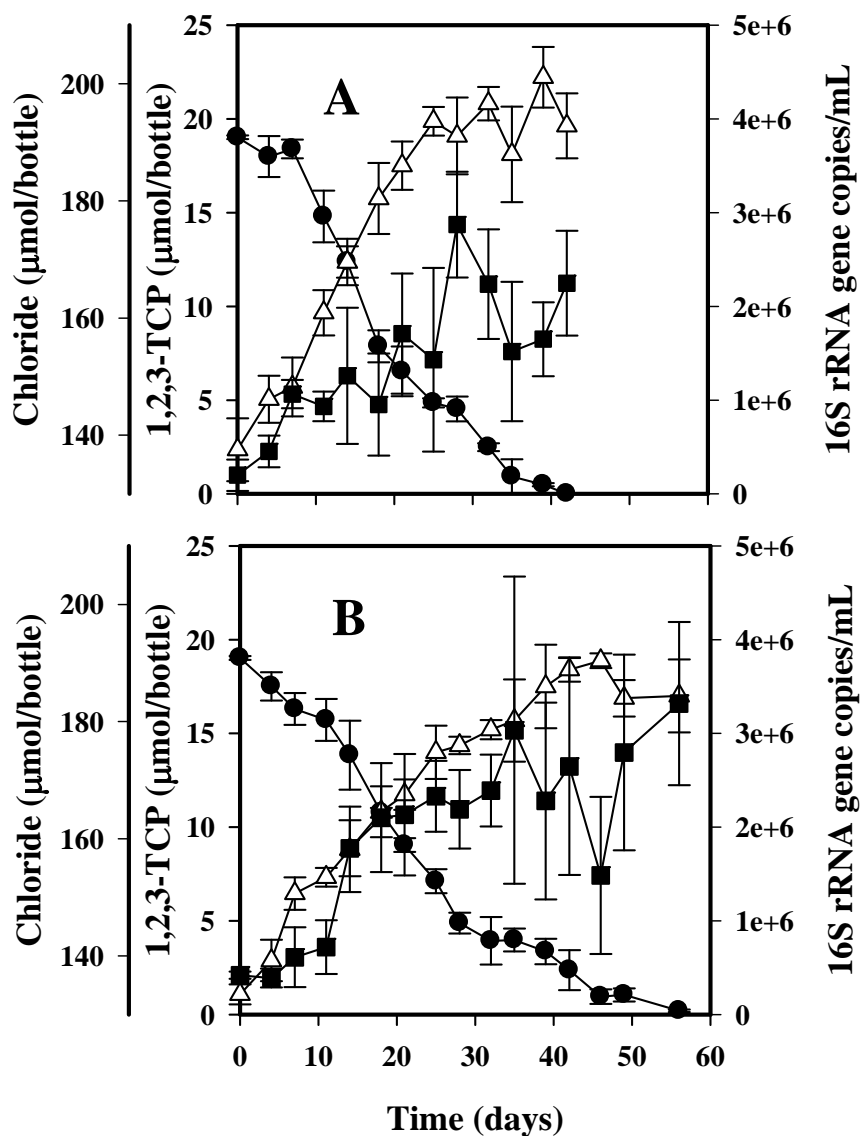


Figure 4.1. Dehalogenation of 1,2,3-TCP by strains BL-DC-9 (A) and BL-DC-8 (B). (●) 1,2,3-TCP; (Δ) chloride; (■) 16S rRNA gene copies. Error bars represent standard deviation of triplicate bottles.

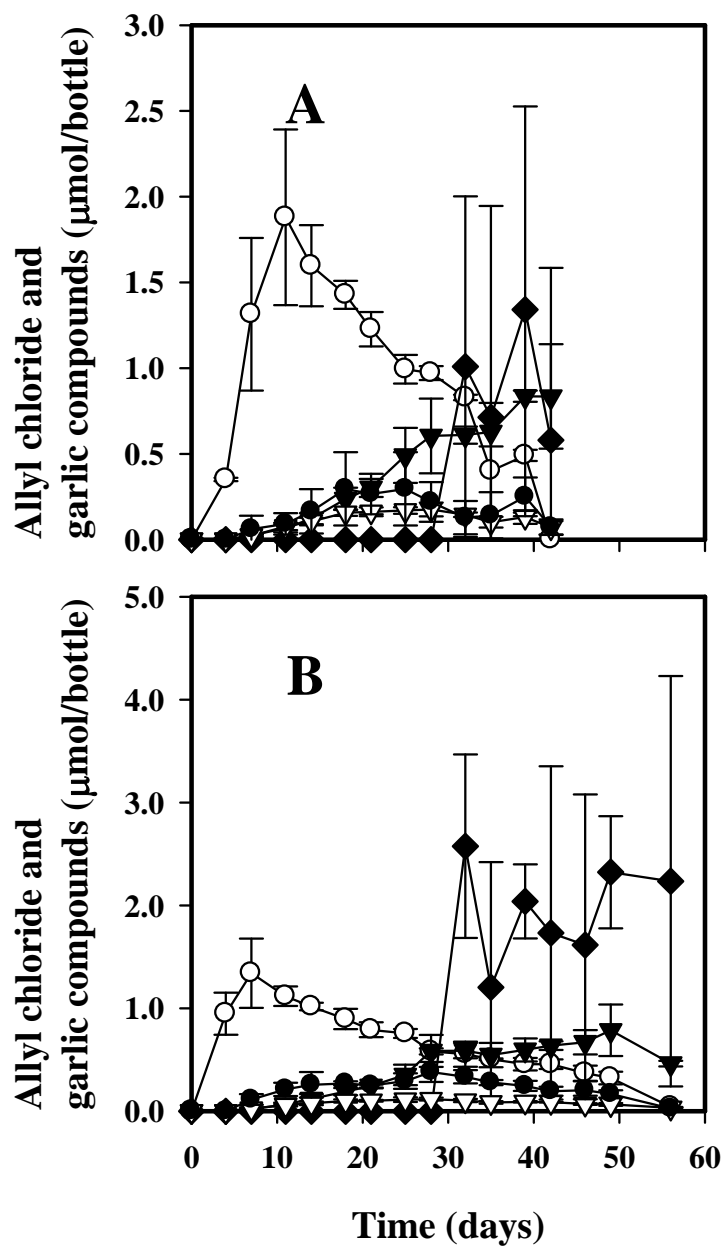


Figure 4.2. Abiotic transformation of allyl chloride during the dehalogenation of 1,2,3-TCP by strains BL-DC-9 (A) and BL-DC-8 (B). (○) allyl chloride; (♦) allyl alcohol; (▼) diallyl sulfide; (◻) diallyl disulfide. Error bars represent standard deviation of triplicate bottles.

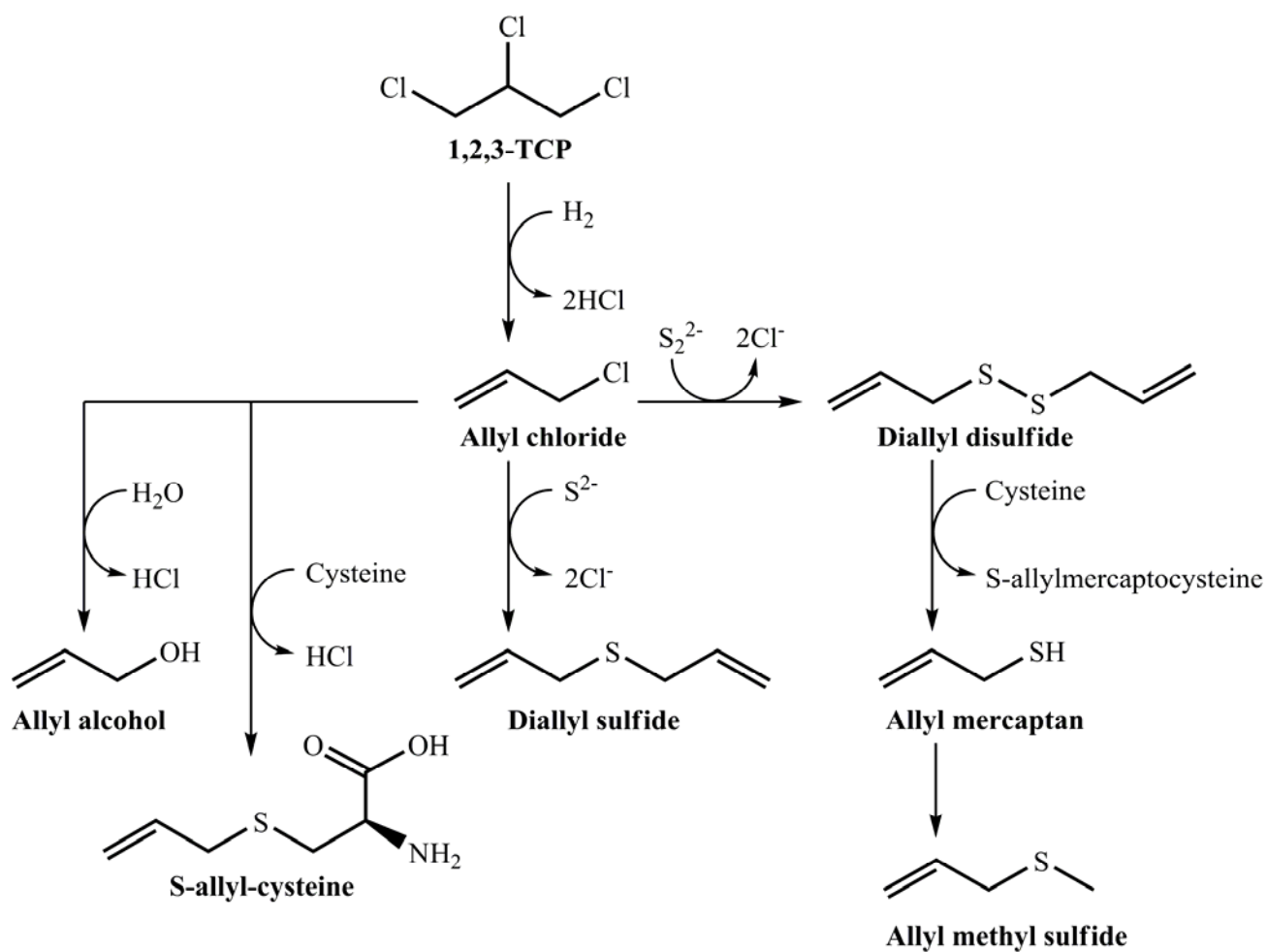


Figure 4.3. Products observed in BL-DC-8 and BL-DC-9 cultures amended with 1,2,3-TCP and the reaction pathways.

1,2,3-TCP has not yet been observed from other bacteria capable of carrying out dihaloelimination reactions, such as *Desulfitobacterium dichloroeliminans* strain DCA1, *Desulfitobacterium* strain Y51 and unknown “Dehalococcoides” strains and *Dehalobacter* strains (Löffler *et al.*, 1997; Suyama *et al.*, 2001; Wildeman *et al.*, 2003; Ritalahti and Löffler, 2004; Grostern and Edwards, 2006).

In the overall 1,2,3-TCP degradation pathway, both biotic and abiotic transformations occurred. The biotic step is reasonably believed to be responsible only for the conversion of 1,2,3-TCP to allyl chloride. A similar biotic dechlorination pattern was observed in dehalogenation of 1,1,2-TCA and 1,1,2,2-TeCA, where the dehalogenation stopped at vinyl chloride, and *cis*- and *trans*-1,2-DCE, respectively. However, unlike vinyl chloride and 1,2-DCEs, allyl chloride is very reactive and can react with numerous compounds including water.

Under aerobic conditions, 1,2,3-TCP was shown to be oxidized by genetically engineered microorganisms that contained a mutated haloalkane dehalogenase (Banáš *et al.*, 2006; Monincová *et al.*, 2007). The final product of 2,3-dichloro-1-propanol, however, is also as recalcitrant and a likely carcinogenic compound as its precursor (Effendi *et al.*, 2000). In contrast, diallyl sulfide, diallyl disulfide, allyl alcohol, and S-allylcysteine, the compounds observed in the anaerobic conditions tested with strains BL-DC-8 and BL-DC-9, are all naturally occurring compounds found in garlic oil and aged garlic extracts (Lawson, 1996). The first three of these compounds, as well as allyl mercaptan which is a metabolite formed in blood following dietary garlic consumption, and allyl methyl sulfide are volatile and contribute to the recognizable garlic odor in exhaled air following garlic consumption (Lawson, 1996; Laasko *et al.*, 1989). From a toxicological perspective, diallyl sulfide, diallyl disulfide, allyl mercaptan, allyl methyl sulfide and S-allylcysteine have been shown to reduce the incidence of chemically induced tumors and proliferation of cancer cells in human and animal models by modulating activity of cytochrome

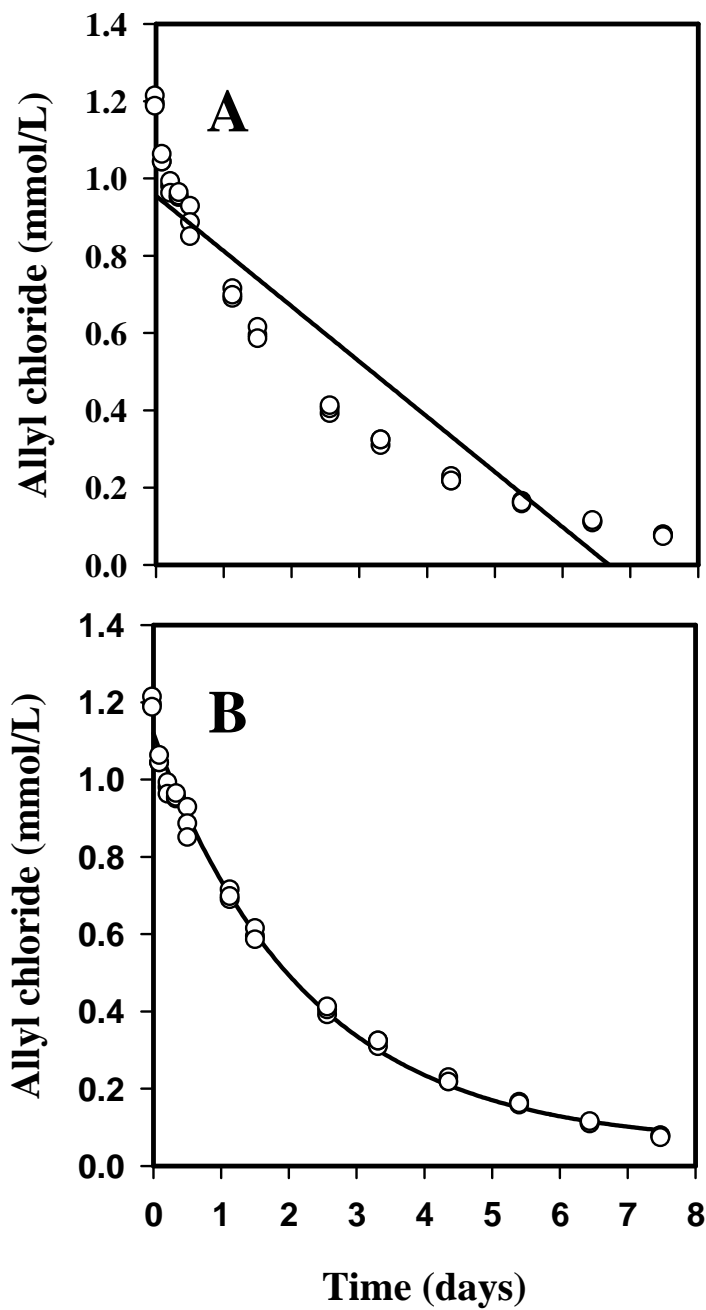


Figure 4.4. Abiotic transformation of allyl chloride was fitted to a simple linear regression model (A) and an exponential decay model (B).

P450 monooxygenases, increasing the activity of phase II detoxification enzymes (e.g., glutathione S-transferases), and inducing apoptosis of cancer cells (Dorai and Aggarwal, 2004).

Allyl alcohol, which is both naturally produced from garlic and synthetically produced as a chemical intermediate in industry, is toxic to fish and aquatic invertebrates (96 hr LC₅₀ of 4.3 µM for *Daphnia magna* (water fleas) and 5.5 µM for *Pimephales promelas* (fathead minnow) (Ewell *et al.*, 1986) as well as laboratory animals (Wistar rats provided drinking water containing ≥ 1.7 mM allyl alcohol for 15 weeks exhibited impaired renal function and increased liver and kidney weights) (Carpanini *et al.*, 1978). The U.S. EPA reference dose for chronic oral exposure is 5×10^{-3} mg/kg/day (www.epa.gov/iris). Although allyl alcohol is hazardous, it is also readily biodegradable under both aerobic and anaerobic conditions (Bridie *et al.*, 1979; Demirer and Speece, 1998) and is unlikely to persist over long time scales in the environment (Krijgheld and van de Gen, 1986). Likewise, the intermediate allyl chloride is also hazardous but unlikely to persist in the environment (Krijgheld and van de Gen, 1986).

4.5 Conclusions

Experimental results demonstrated that 1,2,3-TCP could be reductively dehalogenated in a growth-linked process by strains BL-DC-8 and BL-DC-9. This represents the first study in which 1,2,3-TCP was demonstrated to undergo reductive dehalogenation by pure cultures. Both biotic and abiotic processes were found to be involved in the pathway for complete dehalogenation of 1,2,3-TCP. A number of 1,2,3-TCP degradation intermediates were identified for the first time. Collectively, these findings suggest that anaerobic biological methods may be suitable for remediation of environmental 1,2,3-TCP contamination. Metabolites identified during the course of this study may prove useful as biomarkers to track the biodegradation of 1,2,3-TCP.

CHAPTER 5. DETECTION AND QUANTIFICATION OF DEHALOGENATING BACTERIAL POPULATIONS VIA PCR-BASED PROTOCOLS

5.1 Introduction

In recent years, DNA-based molecular approaches have been increasingly used to detect and quantify “Dehalococcoides” phylotypes in dehalogenating mixed cultures and environmental samples (Löffler *et al.*, 2000; Hendrickson *et al.*, 2002; Cupples *et al.*, 2003; Duhamel *et al.*, 2004; Ritalahti *et al.*, 2006; Cupples, 2008). Previously reported “Dehalococcoides” strains and their environmental clone sequences share high 16S rRNA gene sequences identities (>98%) (Maymo-Gatell *et al.*, 1997; Adrian *et al.*, 2002a; Cupples *et al.*, 2003; He *et al.*, 2003b, 2005; Sung *et al.*, 2006). Current information derived from genome sequences indicates that “Dehalococcoides” strains contain a single 16S rRNA gene copy (Seshadri *et al.*, 2005; Kube *et al.*, 2005). Thus, 16S rRNA gene sequences are generally considered to be a good target for identification and quantification of “Dehalococcoides” populations in mixed microbial communities.

Among the DNA-based tools employed for detection and quantification of “Dehalococcoides”, Hendrickson *et al.* (2002) reported several oligonucleotide primer sequences intended to specifically target variable regions of 16S rRNA genes specific to “Dehalococcoides” strains. Primer specificities were evaluated by checking sizes and sequences of amplicons produced from mixed cultures that fully dehalogenated chlorinated ethenes. These primers were used to investigate environmental distributions of populations in chlorinated solvent contaminated sites throughout North American and Europe. “Dehalococcoides” 16S rRNA genes were subsequently detected from 21 out of 24 sites, including all of the sites where reductive dechlorination proceeded beyond vinyl chloride to ethene. “Dehalococcoides” were not detected using these primers sets in PCR using DNA extracted from samples originating from locations

where complete dechlorination was not observed. This, combined with the fact that PCR amplicons were also detected using these primer sequences in enrichment cultures that completely dehalogenated chlorinated alkenes to ethene but not in enrichment cultures where dechlorination was incomplete, led to the conclusion that the presence of “Dehalococcoides” strains may be a necessary prerequisite for complete reductive dehalogenation of chlorinated ethenes (Hendrickson *et al.*, 2002).

As reported in Chapter 3, “Dehalococcoides” strains are the closest cultured phylogenetic relatives of strains BL-DC-8 and BL-DC-9. Strains BL-DC-8 and BL-DC-9, however, are distantly related to Dehalococcoides (90% 16S rRNA gene sequence identity). Preliminary analysis indicated that the newly isolated strains shared high homology with “Dehalococcoides” in some variable regions of their 16S rRNA gene sequences.

The study reported in this chapter was aimed at: (1) re-evaluating oligonucleotide primers and PCR protocols previously reported as allowing specific detection of “Dehalococcoides” strains 16S rRNA gene sequences, and (2) design and testing of oligonucleotide primers, PCR protocols, and qPCR protocols that allow detection and quantification of strains BL-DC-8 and BL-DC-9 in enrichment cultures as well as in environmental samples.

5.2 Materials and Methods

5.2.1 Evaluation of “Dehalococcoides” 16S rRNA Primer Specificity

Twelve sets of previously published oligonucleotide primers targeting 16S rRNA gene sequences of “Dehalococcoides” strains were evaluated in this study (Table 5.1). These primers had been used previously in PCR, nested-PCR or DGGE-PCR to identify “Dehalococcoides” strains in environmental samples or chloroethene-dehalogenating mixed cultures (Löffler *et al.*, 2000; Hendrickson *et al.* 2002; Dennis *et al.*, 2003; Cupples *et al.*, 2003; Duhamel *et al.*, 2004).

Sequences of these twelve primer sets, comprised of 18 unique primer sequences, were manually checked and aligned against the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9.

To experimentally test if primers intended to target “Dehalococcoides” strains would amplify DNA from strain BL-DC-9, PCR reactions using primer sets listed in Table 5.1 were performed using strain BL-DC-9 genomic DNA as template. The temperature profiles used in PCR reactions were identical to those listed in the references describing the primer sequences. PCR reactions were performed in 25 µL volumes with the same concentrations of forward and reverse primers, Mg^{2+} , dNTP and cycle numbers as specified for these “Dehalococcoides” strains primers in the original publications (Table 5.1). Hendrickson *et al.* (2002) reported a range of 30 or 40 cycles, and the mid-range value of 35 cycles was used for PCR reactions testing the Hendrickson *et al.* primers in this study. AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) was used as the Taq enzyme with activation at 95 °C for 10 min before proceeding to the thermal cycles.

Following PCR, reaction products were electrophoresed in a 3% low melting temperature agarose gel (Genepure Sieve GQA, ISC BioExpress Inc., Kaysville, UT), stained with ethidium bromide (1 µg/mL, BioRad) solution for 15 min, and destained in deionized water for 15 min prior to visualization using a UV transilluminator (ChemIDoc XRS gel documentation system, BioRad). For PCR primers in which amplification products were observed, additional PCR reactions were performed using a plasmid containing a partial 16S rRNA gene insert from BL-DC-9 as the template. This was done to exclude the possibility that contamination from “Dehalococcoides” strains in strain BL-DC-9 genomic DNA might have resulted in PCR product formation. Details regarding the plasmid construction and purification were described previously in section 3.3.11.

For “Dehalococcoides” primer sets that amplified DNA from BL-DC-9 under the PCR

Table 5.1. “Dehalococcoides” 16S rRNA primer sets evaluated in this study.

Primer ID	Primer sequence (5'-3')	Reported T _a (°C) ^a	Reference
Fp DHC 1	GAT GAA CGC TAG CGG CG	55	Hendrickson <i>et al.</i> , 2002
Rp DHC 692	TCA GTG ACA ACC TAG AAA AC		
Fp DHC 1	GAT GAA CGC TAG CGG CG	55	
Rp DHC 1212	GGA TTA GCT CCA GTT CAC ACT G		
Fp DHC 1	GAT GAA CGC TAG CGG CG	55	
Rp DHC 1377	GGT TGG CAC ATC GAC TTC AA		
Fp DHC 385	GGG TTG TAA ACC TCT TTT CAC	55	
Rp DHC 806	GTT AGC TTC GGC ACA GAG AG		
Fp DHC 587	GGA CTA GAG ACA GCA GGA GAA AAC	55	
Rp DHC 1090	GGC AGT CTC GCT AGA AAA T		
Fp DHC 774	GGG AGT ATC GAC CCT CTC	55	
Rp DHC 1212	GGA TTA GCT CCA GTT CAC ACT G		
Fp DHC 946	AGT GAA CCG AAA GGG AAA	55	
Rp DHC 1212	GGA TTA GCT CCA GTT CAC ACT G		
728f	AAG GCG GTT TTC TAG GTT GTC AC	58	Loffler <i>et al.</i> , 2000
1172r	CGT TTC GCG GGG CAG TCT		
1f	GAT GAA CGC TAG CGG CG	59	Duhamel <i>et al.</i> , 2004
259r	CAG ACC AGC TAC CGA TCG AA		
582f	CTG TTG GAC TAG AGT ACA GC	59	
728r	GTG ACA ACC TAG AAA ACC GCC TT		
DeF	GCA ATT AAG ATA GTG GC	55	Cupples <i>et al.</i> , 2003
DeR	ACT TCG TCC CAA TTA CC		
DHE-for	AAG GCG GTT TTC TAG GTT	58	Dennis <i>et al.</i> , 2003
DHE-rev	CGT TTC GCG GGG CAG TCT		

^a: Annealing temperature in the PCR or DGGE-PCR thermal protocol.

reaction conditions described above, additional experiments were performed using the same primer sets and reagent concentrations but at annealing temperatures higher than those reported in Table 5.1. The annealing temperatures were successively increased until no PCR reaction products were detected using BL-DC-9 genomic DNA or plasmid DNA containing a BL-DC-9 partial 16S rRNA gene insert.

5.2.2 Design of 16S rRNA Gene Specific Primers

Oligonucleotide primers targeting 16S rRNA gene sequences unique to strains BL-DC-8 and BL-DC-9 were designed by Brian Rash using consensus variable and hypervariable regions in the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9 (Table 5.2). Candidate primer sequences were selected manually. The uniqueness of candidate sequences was examined by comparison to sequences in the GenBank database and using the Ribosomal Database Project II on-line Probe Match program (<http://rdp.cme.msu.edu/>).

Thirteen primer combinations (Table 5.2) were experimentally tested. In initial tests to verify primer function, genomic DNA from strain BL-DC-9 was used as template in PCR reactions. Each PCR reaction contained 0.5 μ M each primer, 2.5 mM MgCl₂, 100 μ M each deoxynucleotide, and 2 U of *AmpliTaq* Gold in 1 \times PCR gold buffer (Applied Biosystems). The thermal program was initial denaturation at 95 °C for 10 min, followed by 35 cycles of 1 min at 94 °C, 45 sec at 63 °C, and 1 min at 72 °C, and a final extension step of 10 min at 72 °C.

Primer specificity was experimentally tested in several ways. First, primer sets listed in Table 5.2 were utilized in PCR reactions with plasmid DNA containing a partial 16S rRNA gene insert from “*Dehalococcoides*” strain DHC-4 (described in chapter 3) as template using PCR reaction conditions as described above. Genomic DNA from strain BL-DC-9 was used as a positive control for each tested primer set. Following PCR, electrophoresis was performed as described above. No PCR reaction products were detected from PCR reactions using the plasmid

DNA with putative “Dehalococcoides” strain DHC-4 insert.

Additional tests for primer specificity are described in subsequent sections. The tests included (a) size determination and sequencing of products from PCR reactions that used DNA extracts from eight different groundwater samples as templates (Section 5.3.3), (b) size determination of products from PCR reactions that used DNA extracts from a dehalogenating enrichment culture as template (Section 5.3.5), and (c) melting curve analysis following qPCR (Section 5.3.4 and 5.3.5).

5.2.3 Groundwater Collection and DNA Extraction

Groundwater samples were collected from eight wells located in the DNAPL source zone at the PPI Superfund site (well ID nos. W-0627-2, W-0721-1, W-0726-4, W-0820-1, W-0822-3, W-0823-2, W-0825-1, W-0828-1) in August 2006. Prior to sample collection, each well was purged, with more than three well volumes extracted before sample collection. For all wells sampled, groundwater was collected in sterile 1.0 L glass bottles leaving little or no headspace, and samples were placed in a cooler on ice during transport to the laboratory (approximately one hour). Groundwater pH was measured using a portable pH meter (Orion model 290A) at room temperature.

A 30 mL volume of groundwater from each well was transferred to a separate sterile 35 mL polypropylene tube. 10 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added and mixed well. After centrifugation for 10 min at 5000 rpm (3,200×g), supernatant was decanted, and then DNA was extracted and purified as described previously (Bowman *et al.*, 2006).

5.2.4 PCR and Nested PCR

DNA extracted from groundwater collected from one well (W-0823-2) was used as the template in separate PCR reactions using all thirteen primer combinations listed in Table 5.2. Genomic DNA from strain BL-DC-9 was used as a positive control. Following PCR,

electrophoresis and imaging was performed as described in Section 5.3.3. PCR amplicons were purified and sequenced using the forward primer used to produce the initial PCR products in each reaction. For long PCR products (e.g., those produced using the BL-DC-57f/BL-DC-1410r, BL-DC-57f/BL-DC-1351r, BL-DC-117f/BL-DC-1020r, BL-DC-142f/BL-DC-1020r, BL-DC-142f/BL-DC-1243r primer sets), the 536f universal bacterial primer was also used in separate sequencing reactions as described previously (Rainey *et al.*, 1996). Resulting sequences were manually checked and aligned against the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9 using BioEdit v. 7.09 (Hall, 1999).

Additionally, DNA extracted from groundwater collected from all eight groundwater wells was used as template in separate PCR reactions that employed two sets of primers. One set of PCR reactions was performed using the BL-DC-631f and BL-DC-796r primer set targeting strains BL-DC-8 and BL-DC-9 (Table 5.2). A second primer combination, 582f-728r (Duhamel *et al.*, 2004, Table 5.1), was employed to target “Dehalococcoides” strains. PCR reaction conditions were as described previously in Section 5.2.2. The annealing temperatures were 63 °C for reactions using the BL-DC-631f/BL-DC-796r and 56 °C for reactions using the 582f-728r primer set. PCR products were purified, electrophoresed, and visualized as described previously.

For cases where no amplification products were detected in PCR reactions directly using environmental DNA extracted from the groundwater, a nested PCR technique was employed. For nested PCR, initial amplification was performed using the universal bacterial primer set 530f-900r (Lane, 1991, Table 5.3) with PCR reagent composition and temperature program as described above except that the annealing temperature was 56 °C. PCR products were purified using an UltraClean™ PCR Cleanup Kit (MoBio Laboratories, Carlsbad, CA) and then used as template in a second round of PCR amplification using specific primer sets, BL-DC-631f/BL-DC-796r or 582f/728r, under the PCR conditions described above. The resulting PCR products

were then purified, electrophoresed, and imaged as described above.

5.2.5 Quantitative Real-Time PCR (qPCR)

A subset of the primer sets developed for specific detection of bacteria represented by strains BL-DC-8 and BL-DC-9 and primers reported previously for detection of “Dehalococcoides” strains were used in quantitative real-time PCR (qPCR) to evaluate concentrations of putative dehalogenating bacteria in: (a) groundwater samples collected from various wells located at the PPI superfund site, and (b) a 1,1,2-TCA degrading enrichment culture. To allow calculation of the number of putative dehalogenating bacteria relative to the total bacterial population size, universal bacterial primers were also employed in the qPCR studies testing groundwater.

The primer set BL-DC-631f/BL-DC-796r was used to quantify concentrations of the bacterial group represented by strains BL-DC-8 and BL-DC-9. The primer set 582f/728r was used to quantify concentrations of “Dehalococcoides” sp. The universal bacterial primer set Bac1055YF/Bac1392R was used to quantify total 16S rRNA gene copies of bacteria. Primer sequences are listed in Table 5.3.

qPCR was performed using an iCycler iQ PCR thermal cycler detection system (Bio-Rad, Hercules, CA) and the qPCR supermix kit (SYBR GreenER, Invitrogen Inc., Carlsbad, CA). Each 25 μ L qPCR reaction contained: 12.5 μ L 2 \times supermix, 500 nM each primer, 2 μ L DNA extract diluted by a factor of 10, and 10 μ L DNA-free water. PCR tubes were sealed with optically clear cap strips and were placed on ice to maintain enzyme activities before reaction. The PCR thermal protocol was 50 $^{\circ}$ C for 2 min (UDG incubation) and 95 $^{\circ}$ C for 8.5 min (UDG inactivation and DNA polymerase activation), followed by 40 cycles of 95 $^{\circ}$ C for 15 sec and annealing for 60 sec. Annealing temperatures varied between the different primer sets used (Table 5.4). Preliminary experiments demonstrated that the annealing temperatures listed in

Table 5.4 did not produce non-specific products when “Dehalococcoides”-specific primers 582f/728r were used in reactions with BL-DC-9 plasmid as template (data not shown). Likewise, preliminary experiments also demonstrated that the annealing temperatures listed in Table 5.4 did not produce non-specific products when BL-DC-specific primers BL-DC-631f/BL-DC-796r were used in reactions with plasmid DNA containing a “Dehalococcoides” strain DHC-4 partial 16S rRNA gene insert as template (data not shown).

Following amplification, melting curve analysis was performed with 1 min denaturation at 95 °C, 1 min annealing at 55 °C, then 80 cycles of 0.5 °C increments (10 sec each) beginning at 55 °C. DNA samples extracted from groundwater and the mixed culture were analyzed in triplicates for each primer set.

Plasmids containing a partial 16S rRNA gene fragment from strain BL-DC-9 were constructed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Inserts were produced by performing PCR using universal bacterial primers 27f and 1392r with strain BL-DC-9 genomic DNA as template. Following growth of recombinant *E. coli* cells in Luria-Bertani medium (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0) amended with 50 µg/L kanamycin (Luria and Burrous, 1957), plasmid DNA was extracted and purified using an UltraClean Mini Plasmid Prep kit (MoBio Laboratories, Carlsbad, CA). Plasmids carrying an insert comprised of a partial 16S rRNA gene segment amplified from strain DHC-4 were prepared as calibration standards for “Dehalococcoides” strains. For quantification of total bacteria 16S rRNA gene copy numbers, two separate standards were utilized. For one of the standards, genomic DNA from competent *E. coli* (Invitrogen) was extracted and purified using a MoBio Microbial DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA). The other standard used for quantification of total bacteria 16S rRNA gene copy numbers was plasmid DNA carrying an insert comprised of a partial 16S rRNA gene segment amplified from strain BL-DC-9.

Table 5.2. Sequences of BL-DC-8 and BL-DC-9 (BL-DC-) specific primers employed in this study

Primer	Position ^a	Primer sequence (5'-3')	T _m ^b (°C)	Amplicon (bp)	Mismatches ^c (bp)	Proposed T _a (°C)
BL-DC 57f	57-84	GCA AGT CGA ACG GTC TCT CGC	66.5	1330	3	63
BL-DC 1410r	1421-1442	AGG TGT TAC CAA CTT TCA TGA C	58.9		3	
BL-DC 57f	57-84	GCA AGT CGA ACG GTC TCT CGC	66.5	1271	3	63
BL-DC 1351r	1362-1383	AAC GCG CTA TGC TGA CAC GCG T	66.4		6-7	
BL-DC 117f	117-140	GTA ATA GGT AAG TAA CCT GCC CTT	61.2	911	6	63
BL-DC 1020r	1026-1052	ATA GCT CCT GAC TTG ACA GGT GGA TC	66.2		6	
BL-DC 142f	143-170	GTG GGG GAT AAC ACT TCG AAA GAA GTG C	67.5	661	12	63
BL-DC 796r	799-827	ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	70.5		6	
BL-DC 142f	143-170	GTG GGG GAT AAC ACT TCG AAA GAA GTG C	67.5	885	12	63
BL-DC 1020r	1026-1052	ATA GCT CCT GAC TTG ACA GGT GGA TC	66.2		6	
BL-DC 142f	143-170	GTG GGG GAT AAC ACT TCG AAA GAA GTG C	67.5	1093	12	63
BL-DC 1243r	1253-1275	CCG GTG GCA ACC CAT TGT ACC GC	69.9		7	
BL-DC 183f	184-210	GGT GCT CTT TCA CAA GGA AGA GTA CTG	66.1	620	13	63
BL-DC 796r	799-827	ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	70.5		6	
BL-DC 610f	613-642	TCT CCC GGC TCA ACT GGG AGG GGT CAT CTG	74.2	439	6	63
BL-DC 1020r	1026-1052	ATA GCT CCT GAC TTG ACA GGT GGA TC	66.2		6	
BL-DC 610f	613-642	TCT CCC GGC TCA ACT GGG AGG GGT CAT CTG	74.2	647	7	63
BL-DC 1243r	1253-1275	CCG GTG GCA ACC CAT TGT ACC GC	69.9		6	
BL-DC 727f	730-751	GAA GGC GGT TTT CTA GGC CAW A ^d	62.7	322	5	63
BL-DC 1020r	1026-1052	ATA GCT CCT GAC TTG ACA GGT GGA TC	66.2		6	
BL-DC 727f	730-751	GAA GGC GGT TTT CTA GGC CAW A	62.7	636	5	63
BL-DC 1351r	1362-1383	AAC GCG CTA TGC TGA CAC GCG T	66.4		6-7	
BL-DC 631f	634-663	GGT CAT CTG ATA CTG TTG GAC TTG AGT ATG	66.0	194	7	63
BL-DC 796r	799-827	ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	70.5		6	
BL-DC 727f	730-751	GAA GGC GGT TTT CTA GGC CAW A	62.7	278	5	63
BL-DC 982r	986-1007	TCT AAC ATG TCA AGC CCT GGT G	62.7		7-8	

^a: Base position number corresponds to *E. Coli* 16S rRNA gene sequence coordinates (GenBank accession number E05133).

^b: Reference melting temperatures were provided by OPERON using the calculation method described in Appendix A.

^c: Base mismatches by aligning with “Dehalococcoides” strains CBDB1, 195, VS, BAV1 and FL2.

^d: W = A or T.

Table 5.3. Primers and PCR conditions in the nested-PCR and qPCR

Primer	Sequence (5'-3')	Target gene	Amplicon (bp)	T _a (°C)	Application	Reference
27f 1392r	GAG TTT GAT CCT GGC TCA ACG GGC GGT GTG TAC	Bacterial 16S rRNA	1354	56	Plasmid construction	Lane, 1991
530f ^a 900r	GTG CCA GCM GCC GCG G CCT TTG AGT TTT AAT CTT	Bacterial 16S rRNA	370	56	Nested PCR	
Bac1055YF ^b Bac1392R	ATG GYT GTC GTC AGC T ACG GGC GGT GTG TAC	Bacterial 16S rRNA	352	64	qPCR	Ritalahti <i>et al.</i> , 2006
BL-DC- 631f BL-DC- 796r	GGT CAT CTG ATA CTG TTG GAC TTG AGT ATG ACC CAG TGT TTA GGG CGT GGA CTA CCA GG	BL-DC- 16S rRNA	194	63/ 68	Nested PCR/ qPCR	This study
582f 728r	CTG TTG GAC TAG AGT ACA GC GTG ACA ACC TAG AAA ACC GCC TT	“Dehalococcoides” 16S rRNA	108	56/ 65	Nested PCR/ qPCR	Duhamel <i>et al.</i> , 2004

^a: M = C or A.

^b: C was modified to Y (= C or T) as recommended by Ritalahti *et al.* (2006).

Concentrations of genomic DNA and plasmids were measured with a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA) at 260 nm. A conversion factor of 50 µg/mL double strand DNA corresponding to an absorbance of 1.0 was applied in the calculation. Serial dilutions of DNA standards were made with a factor of 10 and the threshold cycle values at each dilution level were obtained in the qPCR reactions. Calibration curves were prepared by plotting the log-transformed gene copy number against the threshold cycle values. The 16S rRNA gene copy number in a qPCR reaction was calculated as described by Ritalahti *et al.* (2006) in equation 1.

$$\begin{aligned} \text{gene copies/reaction} = & (2 \text{ } \mu\text{L/reaction}) (\text{DNA concentration}[\text{ng}/\mu\text{L}]) \left(\frac{1 \text{ g}}{1,000^3 \text{ ng}} \right) \left(\frac{1 \text{ mol bp DNA}}{660 \text{ g DNA}} \right) \\ & \times \left(\frac{6.023 \times 10^{23} \text{ bp}}{\text{mol bp}} \right) \left(\frac{1 \text{ copy}}{\text{genome or plasmid size}[\text{bp}]} \right) \quad (1) \end{aligned}$$

Plasmid DNA carrying a partial 16S rRNA gene insert from strain BL-DC-9 was to prepare calibration curve for total bacteria 16S rRNA gene copy numbers using primers Bac1055YF/Bac1392R. The calibration curves prepared using plasmid DNA assumed a plasmid size of 5308 bp [pCR 4-TOPO plasmid (3954 bp) (Invitrogen Co., Carlsbad, CA) plus inserted sequences (1354 bp) for both DC-9 and DHC-4] and one copy of the 16S rRNA gene per plasmid DNA-free water or plasmids without target gene inserts were used as negative controls. PCR amplification efficiency was calculated by equation 2.

$$\eta = 10^{\left(-\frac{1}{\text{slope}}\right)} - 1 \quad (2)$$

Each calibration curve was prepared using three independent serial dilutions of DNA standards. A simple linear regression model was built using SAS 9.0 to calculate slope, intercept,

correlation coefficient (R^2), and standard deviation of each parameter.

The qPCR analysis protocol described above was applied to groundwater community DNA extracted from samples collected from eight wells located in the DNAPL source zone of the PPI site (described in Section 5.3.5). This was intended to allow assessment of the relative abundance of putative dechlorinating bacteria at the site at a single point in time. The qPCR analysis protocol was also applied to study the relative abundance of putative dechlorinating bacteria over time in an enrichment culture described below in Section 5.3.6.

5.2.6 Enrichment Culture Experiments

The 1,1,2-TCA enrichment culture described in Chapter 2 as the starting culture used for isolation of dehalogenating bacteria was maintained via successive transfers over a period lasting more than three years. Complete dehalogenation of 1,1,2-TCA to ethene was consistently observed with vinyl chloride as the only intermediate. No methane production was observed in this culture. 10% (v/v) transfers into fresh basal medium amended with 1,1,2-TCA were made at approximately two-month intervals after 1,1,2-TCA was completely transformed to ethene.

The experiment to track relative abundance of various dechlorinating bacterial populations as a function of time in the enrichment culture was conducted in sealed serum bottles (68 mL) containing 38.8 mL basal medium and gas mixture comprised of $H_2/CO_2/N_2$ (10/10/80, v/v/v) in the headspace. The composition and preparation of anaerobic basal medium were described in section 2.2.3. Each bottle was spiked with 1.9 μ L neat 1,1,2-TCA, resulting in an initial aqueous-phase concentration of approximately 0.5 mM after dissolution and equilibration. Each bottle received 1.2 mL inoculum (3%, v/v) from the previously maintained 1,1,2-TCA enrichment culture. Triplicate bottles were sacrificed twice per week for analysis of 1,1,2-TCA and degradation products (using GC methods described in Chapter 2) as well as gene copy

numbers. Community DNA was extracted from the cell pellet from 15 mL aliquots of culture volume as described in Chapter 2. Ultra-high-purity filter-sterilized hydrogen was amended (resulting in 5% v/v H₂ in the headspace) when dehalogenation was found stopped at day 31.

In a preliminary experiment using DNA extracted from bottles sacrificed on days 49, PCR reactions were performed using the primer set DHB477f/DHC647r (Grostern and Edwards, 2006) which was reported previously to target 16S rRNA gene sequences specific to *Dehalobacter* sp., another known dehalogenating group of bacteria. PCR reactions used the same composition and thermal cycle program as reported by Grostern and Edwards (2006) for this primer set. No PCR amplification was detected, and this primer set was not employed in the qPCR experiments.

5.2.7 Analytical Methods

Quantitative analysis of 1,1,2-TCA, vinyl chloride and ethene were performed with an HP model 6890 gas chromatograph equipped with a flame ionization detector (FID) and GS-GasPro capillary column (60 m × 0.32 mm I.D., J&W P/N 113-4362) as described in Chapter 2. Headspace hydrogen concentration was measured using a 0.1 mL gastight syringe (Teklab, Baton Rouge, LA) and a gas chromatograph (model 310; SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector and a molecular sieve column (Alltech Molesieve 5A 80/100) with nitrogen as the carrier gas. The temperature of the injection port was 100°C, the oven 50 °C, and the detector 100 °C, as described by van Ginkel *et al.* (2001).

5.3 Results

5.3.1 Specificities of “Dehalococcoides” 16S rRNA Primers

As described in Section 5.2.1, the sequences of twelve “Dehalococcoides” primer sets (comprised of a total of 18 unique primer sequences) were manually checked and aligned against

16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9. “Dehalococcoides” primers Fp DHC 1, Fp DHC 774 and Rp DHC 806 (Hendrickson *et al.*, 2002) were found to exactly complement the corresponding binding regions in the 16S rRNA sequences of strains BL-DC-8 and BL-DC-9. An additional “Dehalococcoides” forward primer, Fp DHC 385, contained only one base mismatch from the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9. Among these 12 primer sets examined, Fp DHC 385/Rp DHC 806 (Hendrickson *et al.*, 2002) had the lowest total number of mismatches, with only 1 base different from the 16S rRNA gene sequence of strains BL-DC-8 and BL-DC-9. The primer sets 728f/1172r (Löffler *et al.*, 2000) and 582f/728r (Duhamel *et al.*, 2004) had the highest total combined mismatches with 8 nucleotide positions differing from the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9.

Electrophoresis of PCR products resulting from reactions employing the reportedly “Dehalococcoides” specific primers in combination with strain BL-DC-9 genomic DNA as template revealed that three of the “Dehalococcoides” primers sets amplified DNA from strain BL-DC-9 (Fig. 5.1 panel A). Strong bands are clearly present for the Fp DHC 385/ Rp DHC 806 and Fp DHC 587/ Rp DHC 1090 primer sets (Fig. 5.1 panel A, lanes 5 and 6). A faint but readily visible band was produced by the Fp DHC 1/ Rp DHC 692 primer set (Fig. 5.1 panel A, lane 2). PCR amplicons observed in the agarose gel are of the sizes expected from amplification of partial 16S rRNA gene segments based on the 16S rRNA sequences of strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” strains. These results demonstrated that some of the “Dehalococcoides”-specific primer sets reported previously in conjunction with their reported PCR temperature programs can amplify strain BL-DC-9’s 16S rRNA gene. If such a protocol were used to establish whether “Dehalococcoides” strains are present in a particular sample, an

erroneous conclusion might be reached.

PCR reactions were performed with annealing temperatures higher than those reported by Hendrickson *et al.* (2002) were tested to determine if non-specific amplification of BL-DC-9 DNA could be eliminated by simply modifying the PCR thermal conditions. Annealing temperatures of 68 °C, 66 °C and 62 °C for primers sets Fp DHC 385/ Rp DHC 806, Fp DHC 587/ Rp DHC 1090 and Fp DHC 1/ Rp DHC 692, respectively, did not result in PCR amplification of the BL-DC-9 genomic DNA template (Fig. 5.1 panel B, lanes 2, 5, 8). Additional verification that the elevated annealing temperatures listed above do not cause non-specific amplification of the bacterial group represented by strains BL-DC-8 and BL-DC-9 comes from the fact that PCR amplicons were also not observed in PCR reaction conducted under the same temperature conditions but using plasmid containing BL-DC-9 partial 16S rRNA gene insert as the template (Fig. 5.1 panel B, lanes 3, 6, 9). This latter test was conducted to eliminate the possibility that genomic DNA from strain BL-DC-9 used for the initial test was contaminated with DNA from “Dehalococcoides” strains. PCR reactions performed at the elevated annealing temperatures did, however, produce amplicons in reactions using plasmid DNA containing a partial 16S rRNA insert from “Dehalococcoides” strain DHC-4 as the template (Fig. 5.1 panel B, lanes 4, 7, 10). This suggests that use of the primer sets can be used to provide specific detection of “Dehalococcoides” strains if the annealing temperature is sufficiently high.

5.3.2 Development of PCR Assays for Strains BL-DC-8 and BL-DC-9

Thirteen primer combinations intended to specifically target 16S rRNA gene sequences of the bacterial group represented by strains BL-DC-8 and BL-DC-9 were tested with strain BL-DC-9 genomic DNA as template. All primer sets produced bright bands with expected sizes in

the resulting electrophoresis gel (Fig. 5.2, panel A). No bands, however, were observed in lanes containing PCR reaction products in which the plasmid with a partial 16S rRNA insert from “Dehalococcoides” strain DHC-4 served as template under identical PCR amplification conditions (Fig. 5.2, panel B). This indicates that the primer sets intended to target only bacteria sharing high similarity with strains BL-DC-8 and BL-DC-9 did not amplify DNA from “Dehalococcoides” strains.

Primer specificities were further evaluated using DNA extracted from groundwater collected from well W-0823-2 in the DNAPL source zone at the PPI Superfund site. All thirteen primer sets listed in Table 5.2 yielded PCR products with sizes expected based on the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9 (Fig. 5.3).

5.3.3 Detection of Dehalogenating Bacterial Populations in Environmental Samples

Primer sets BL-DC-631f/BL-DC-796r and 582f/728r were selected to identify the presence of strains BL-DC-8, BL-DC-9, and “Dehalococcoides” strains in DNA extracts from groundwater samples collected from the DNAPL source zone at the PPI Superfund site. Direct (i.e., non-nested) PCR yielded bands corresponding to BL-DC-8 and BL-DC-9 from only two samples (W-0721-1 and W-0823-2). Bands corresponding to “Dehalococcoides” strains were also detected from only two samples (W-0820-1 and W-0823-2) using direct (i.e., non-nested) PCR (data not shown). Increasing DNA template volumes or diluting DNA template concentrations did not produce additional positive results.

In the nested-PCR approach with initial amplification using the universal bacterial primer set 530f/900r (Table 5.3), PCR products with the expected size were produced from seven groundwater DNA samples. Amplification of DNA from well W-0828-1 was unsuccessful (data not shown). The PCR products from the seven samples in which amplicons were observed were

Table 5.4. Specificity test for selected “Dehalococcoides”-specific 16S rRNA gene primer sets

Primer	T _m ^a (°C)	Amplicon (bp)	Mismatches ^b (bp)	Reported T _a (°C)	False positive ^c	Revised T _a (°C)	Verified ^d	Reference
Fp DHC 1 Rp DHC 692	62.0 56.3	692	0 5	55	+	62	Yes	Hendrickson <i>et al.</i> , 2002
Fp DHC 1 Rp DHC 1212	62.0 62.7	1212	0 4	55	-	/	Yes	
Fp DHC 1 Rp DHC 1377	62.0 60.4	1377	0 4	55	-	/	N/A	
Fp DHC 385 Rp DHC 806	58.7 62.4	421	1 0	55	+	68	Yes	
Fp DHC 587 Rp DHC 1090	64.6 58.0	503	3 2	55	+	66	Yes	
Fp DHC 774 Rp DHC 1212	62.2 62.7	438	0 4	55	-	/	Yes	
Fp DHC 946 Rp DHC 1212	55.3 62.7	266	2 4	55	-	/	Yes	
728f 1172r	62.8 64.5	444	5 3	58	-	/	Yes	Löffler <i>et al.</i> , 2000
1f 259r	62.0 62.4	258	0 2	59	-	/	Yes	Duhamel <i>et al.</i> , 2004
582f 728r	60.4 62.8	108	3 5	59	-	/	Yes	
DeF DeR	52.4 54.8	1373	3 2	55	-	/	N/A	Cupples <i>et al.</i> , 2003
DHE-for DHE-rev	62.8 64.5	443	2 3	58	-	/	Yes	Dennis <i>et al.</i> , 2003

^a: Reference melting temperatures were provided by OPERON using the calculation method described in Appendix A.

^b: Number of nucleotide mismatches in each primer relative to strain BL-DC-9.

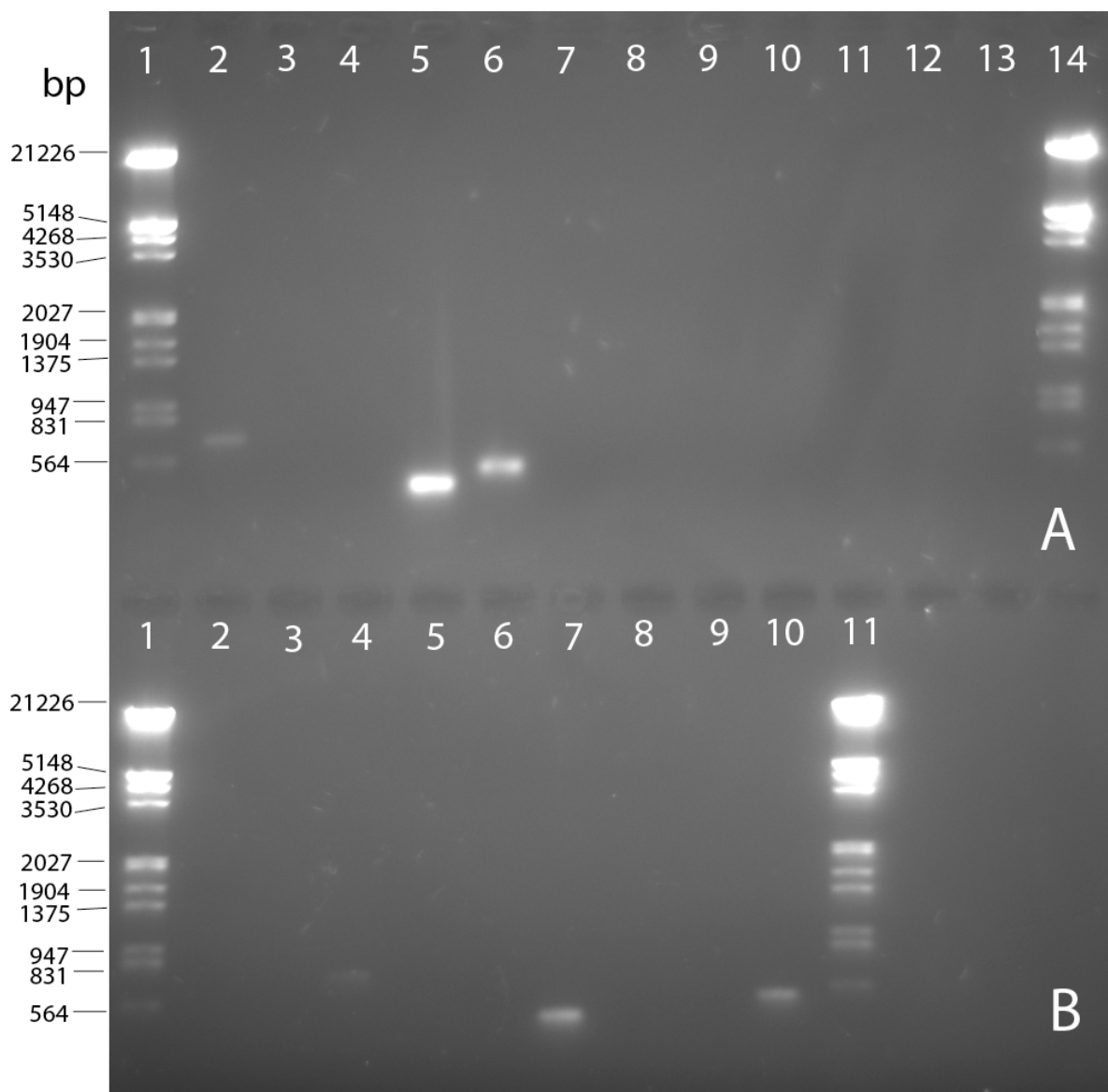
^c: “False positive” indicates that the primer set resulted in amplification when genomic DNA from strains BL-DC-8 and BL-DC-9 was used as template.

^d: “Verified” indicates that false positive was confirmed by using plasmid DNA containing a partial 16S rRNA gene sequence from strain BL-DC-9 as the template under reported T_a. N/A indicated the primers were not covered by the inserted 16S rRNA sequence and could not be verified.

Figure 5.1. Specificity test of “Dehalococcoides” 16S rRNA gene primer sets.

Panel A, lanes 1 and 14, DNA marker III 0.12~21.2 kbp (Roche, Germany); lane 2, Fp DHC 1/ Rp DHC 692; lane 3, Fp DHC 1/ Rp DHC 1212; lane 4, Fp DHC 1/ Rp DHC 1377; lane 5, Fp DHC 385/ Rp DHC 806; lane 6, Fp DHC 589/ Rp DHC 1090; lane 7, Fp DHC 774/ Rp DHC 1212; lane 8, Fp DHC 946/ Rp DHC 1212; lane 9, 728f-1172r; lane 10, 1f-259r; lane 11, 582f-728r; lane 12, DeF-DeR; lane 13, DHE-for-DHE-rev. The amplicons imaged in gel part A were generated using strain BL-DC-9 genomic DNA as template with an annealing temperature of 55°C.

Panel B, lane 1 and 11, DNA marker III; lanes 2, 3 and 4, Fp DHC 1/ Rp DHC 692 tested with BL-DC-9 genomic DNA, plasmid DNA containing BL-DC-9 insert, and plasmid DNA containing DHC-4 insert as template, respectively, with PCR annealing temperature at 62°C; lane 5, 6 and 7, Fp DHC 385/ Rp DHC 806 tested with BL-DC-9 genomic DNA, plasmid DNA containing BL-DC-9 insert, and plasmid DNA containing DHC-4 insert as template, respectively, with PCR annealing temperature at 68°C; lane 8, 9 and 10, Fp DHC 587/ Rp DHC 1090 tested with BL-DC-9 genomic DNA, plasmid containing BL-DC-9 insert, and plasmid containing DHC-4 insert, respectively, with PCR annealing temperature at 66°C.



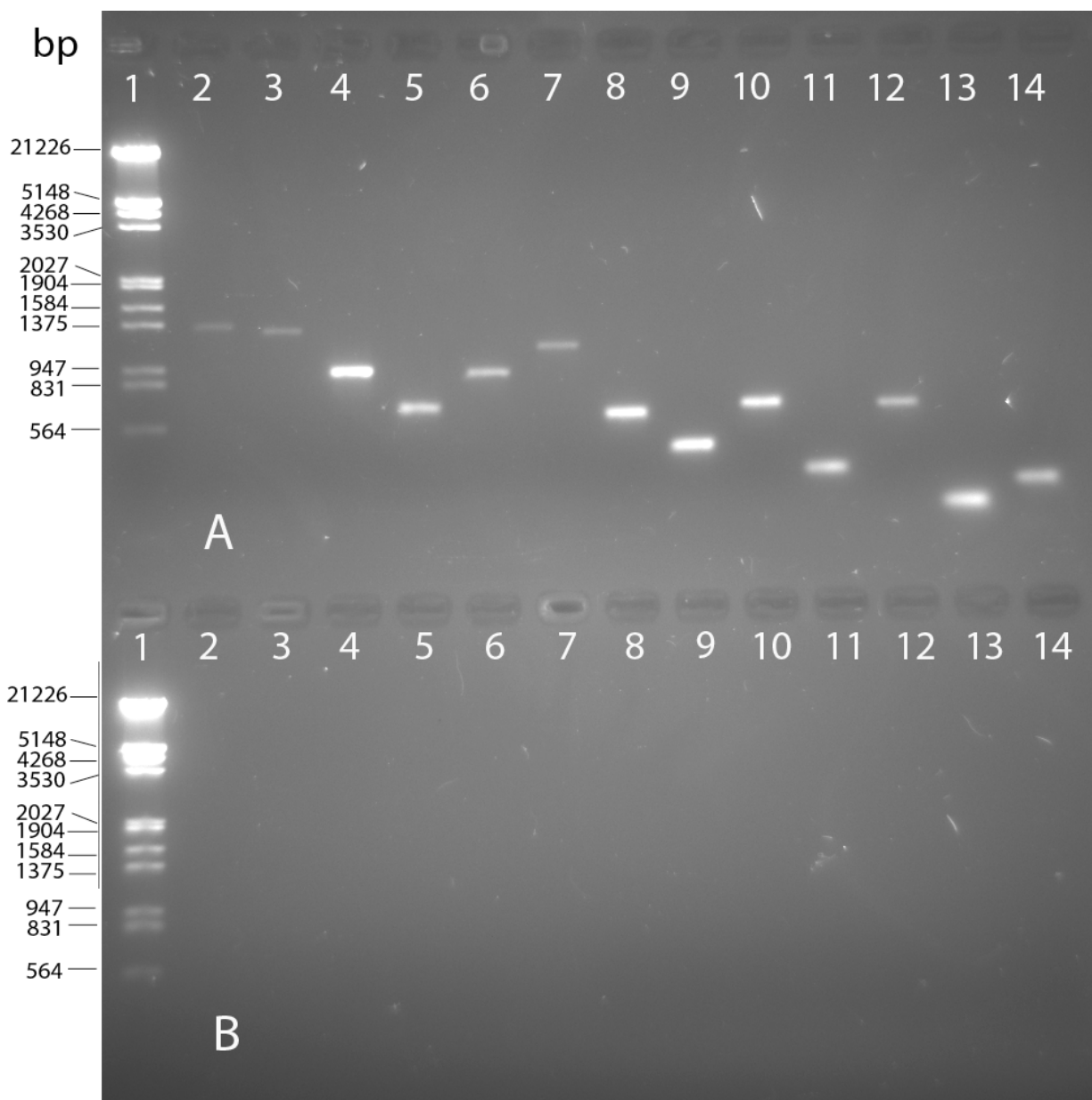


Figure 5.2. Specificity test of BL-DC- primer sets with strain BL-DC-9 genomic DNA as template (panel A). lane 1, DNA marker III 0.12~21.2 kbp (Roche, Germany); lane 2 to 14, BL-DC-57f/BL-DC-1410r, BL-DC-57r/BL-DC-1352r, BL-DC-117f/BL-DC-1020r, BL-DC-142f/BL-DC-796r, BL-DC-142F/BL-DC-1020R, BL-DC-142f/BL-DC-1243r, BL-DC-183f/BL-DC-796r, BL-DC-610f/BL-DC-1020r, BL-DC-610f/BL-DC-1242r, BL-DC-727f/BL-DC-1020r, BL-DC-727f/BL-DC-1351r, BL-DC-631f/BL-DC-796r, BL-DC-727f/BL-DC-982r. Test of BL-DC-primer sets with plasmid containing a partial 16S rRNA gene insert from “Dehalococcoides” strain DHC-4 (panel B). lane 1, DNA marker III 0.12~21.2 kbp (Roche, Germany); lanes 2 to 14, BL-DC- primer sets in the same order in panel A.

purified and then used as templates for PCR reactions using primer sets BL-DC-631f/BL-DC-796r or 582f/728r. After the second-round of amplification, PCR products corresponding to strains BL-DC-8 and BL-DC-9 16S rRNA gene sequences were detected in all seven groundwater samples (Fig. 5.4 panel A, lanes 2-8). “Dehalococcoides” sp. 16S rRNA gene sequences were detected in six samples (Fig. 5.4 panel B, lanes 3-8). No amplification products were observed in the DNA extracts from well W-0627-2 (Fig. 5.4 lane 2). All positive bands observed in electrophoresis gels following the second-round PCR were of the sizes expected based on 16S rRNA gene sequences of strains BL-DC-8, BL-DC-9, and “Dehalococcoides” strains.

The PCR products resulting from the second-round of amplification in the nested PCR procedure using primer sets BL-DC-631f/BL-DC-796r or 582f/728r were purified and sequenced. The resulting sequences were aligned against strain BL-DC-9 16S rRNA gene sequences and “Dehalococcoides” strains BAV1, FL2, CBDB1 and 195 (GenBank accession numbers AY165308, AF357918, AF230641, and AF004928). Sequences of amplicons generated using “Dehalococcoides” specific primer set 582f/728r completely matched the sequences of “Dehalococcoides” strains BAV1, FL2, CBDB1 and 195, which are identical over the range amplified by the primer combination. Sequences of amplicons generated using the primer set BL-DC- 631f/BL-DC-796r from groundwater DNA samples W-0721-1, W-0726-4, W-0822-3, W-0823-2, W-0820-1, and W-0825-1 were identical to the 16S rRNA gene sequence of strain BL-DC-9. The sample from well W-0627-2 had three base mismatches with strain BL-DC-9, and seven base mismatches with “Dehalococcoides” strain BAV1.

Collectively, these data indicate that the primer sets employed for the experiment are specific to the bacterial groups that they were intended to target. These data also indicate that the

bacterial population represented by strains BL-DC-8, BL-DC-9 and “Dehalococcoides” strains are widely distributed throughout the DNAPL source zone of the Brooklawn portion of the PPI Superfund site.

5.3.4 qPCR Calibration Curves

Primer sets BL-DC-631f/BL-DC-796r and 582f/728r were used in qPCR reactions to quantify the 16S rRNA genes of strains BL-DC-8, BL-DC-9 and “Dehalococcoides” strains, respectively. Calibration curves generated by amplifying serially diluted plasmids containing corresponding 16S rRNA gene inserts are shown in Fig. 5.5 and 5.6. Presence of only single PCR products in both qPCR reactions were verified with melting curve analysis. The melting temperatures of 16S rRNA qPCR products were found to be 86 °C and 81 °C for strain BL-DC-9 and “Dehalococcoides”, respectively. A universal bacterial primer set Bac1055YF/Bac1392R was used to target bacterial 16S rRNA genes.

The qPCR reaction parameters listed in Table 5.6 were calculated using a simple linear regression model constructed with SAS 9.0. The slope of the log-transformed calibration curve was used to calculate the PCR amplification efficiency using equation 6-2. A perfect PCR amplification efficiency of 100% corresponds to the case where the concentration of PCR products doubles after each PCR thermal cycle, and corresponds to a slope of 3.32. The PCR efficiency using primer set BL-DC-631f/ BL-DC-796r with plasmid DNA containing a partial 16S rRNA gene insert from strain BL-DC-9 ranged from 100%-102%. PCR amplification efficiency using universal bacterial primers Bac1055YF/ Bac1392R varied from 94% to 102% (corresponding to calibration curve slope of -3.37 ± 0.10), which is consistent with the previously reported value (slope of -3.34 ± 0.14) using the same primer set and plasmid DNA containing “Dehalococcoides” strain FL2 16S rRNA gene insert as template (Ritalahti *et al.*, 2006). For

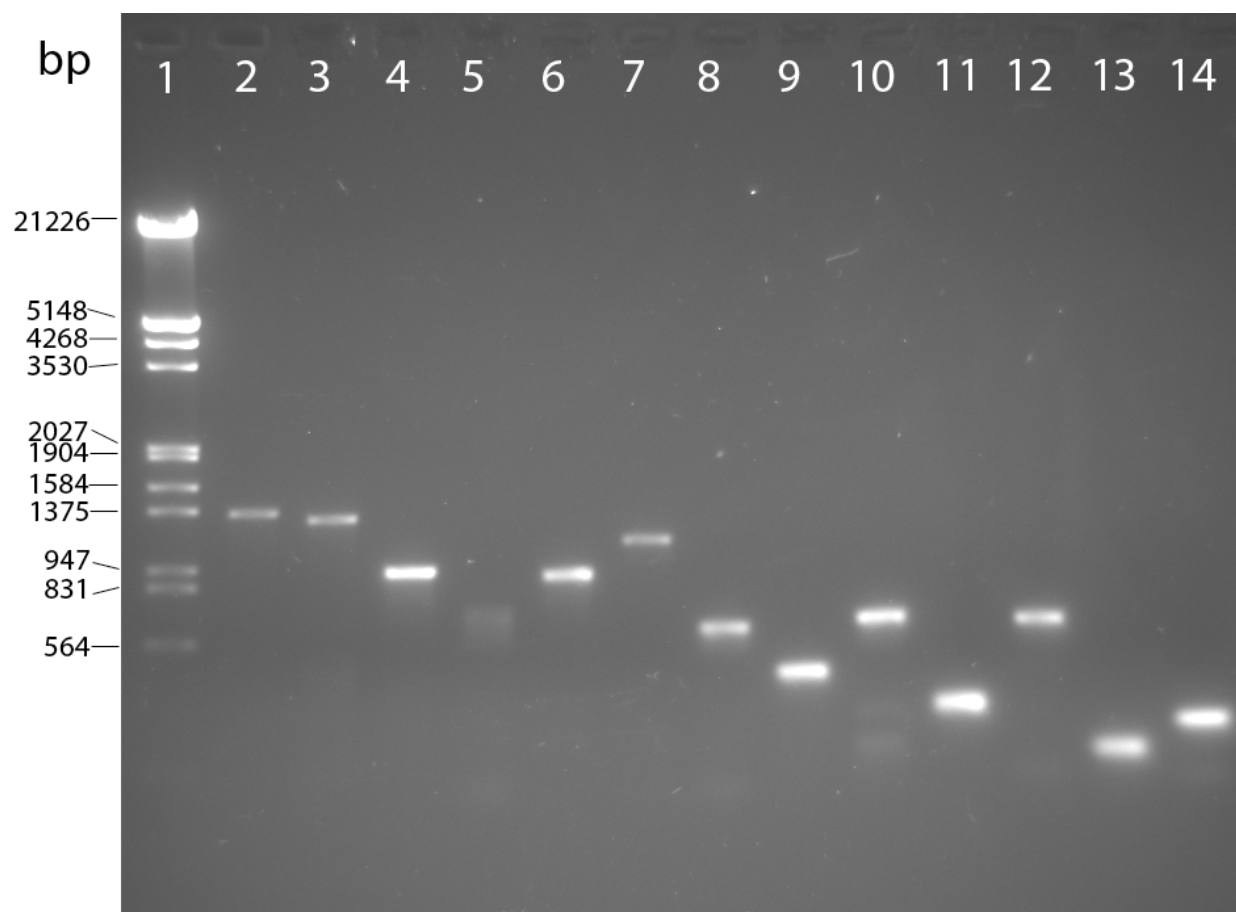


Figure 5.3. PCR amplification of DNA extract from groundwater sample collected from well ID W-0823-2 using BL-DC- specific primer sets. Lane 1, DNA marker III 0.12~21.2 kbp (Roche, Germany); lane 2-14, PCR products generated with primer sets BL-DC-57f/BL-DC-1410r, BL-DC-57f/BL-DC-1351r, BL-DC-117f/BL-DC-1020r, BL-DC-142f/BL-DC-796r, BL-DC-142f/BL-DC-1020r, BL-DC-142f/BL-DC-1243r, BL-DC-183f/BL-DC-796r, BL-DC-610f/BL-DC-1020r, BL-DC 610f/BL-DC-1243r, BL-DC-727f/BL-DC-1020r, BL-DC-727f/BL-DC-1351r, BL-DC-631f/BL-DC-796r, and BL-DC-727f/BL-DC-982r.

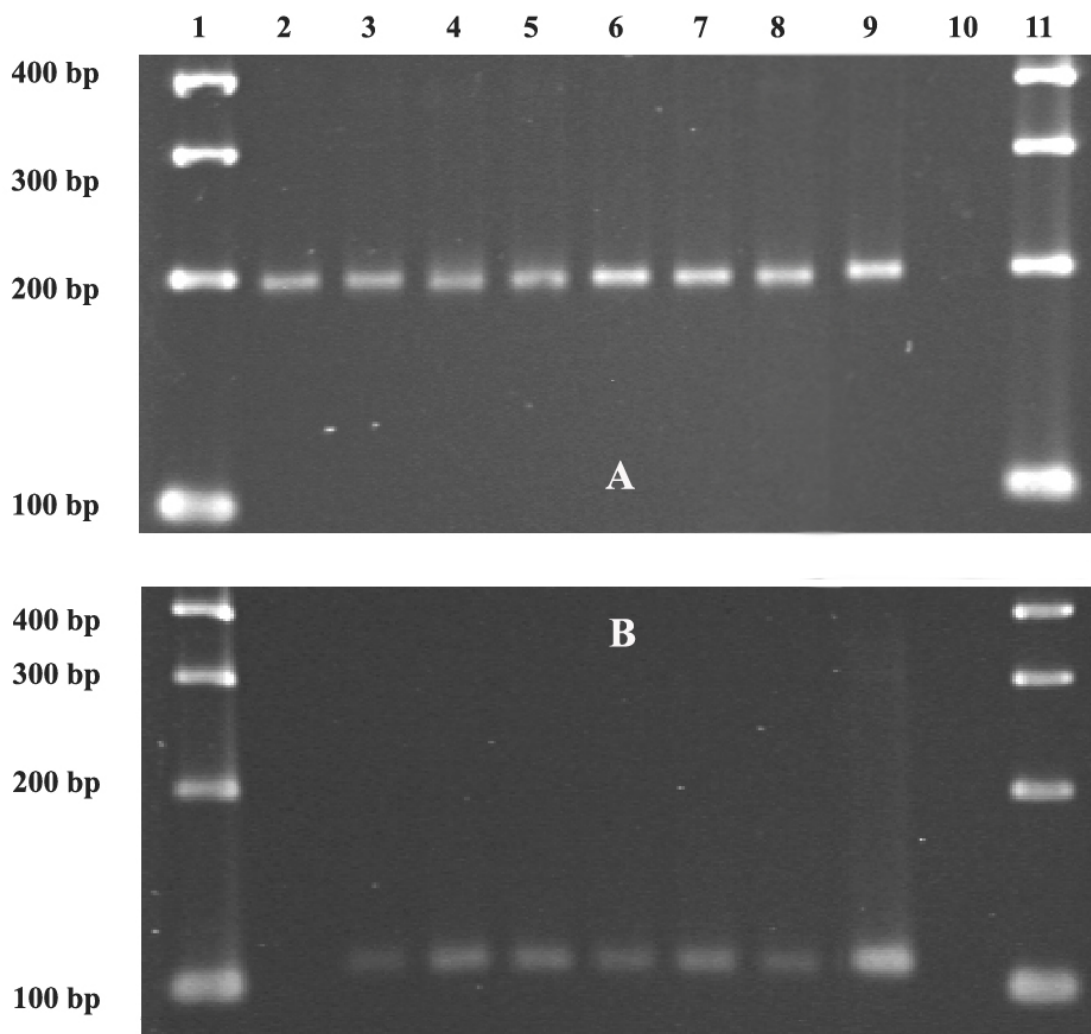


Figure 5.4. Detection of BL-DC- 16S rRNA gene sequences (A) and “Dehalococcoides” 16S rRNA gene sequences (B) with nested PCR. Lane 1 and 11, DNA ladder (100 bp DNA ladder, Biolabs Inc.); lanes 2 to 8, PCR products from wells W-0627-2, W-0721-1, W-0726-4, W-0822-3, W-0823-2, W-0820-1, W-0825-1; lane 9, strain BL-DC-8 genomic DNA as positive control for BL-DC- primers (A) genomic DNA from putative “Dehalococcoides” strain DHC-4 as positive control for DHC primers (B); lane 10, sterile water used as negative control.

“Dehalococcoides” 16S rRNA gene calibration curve, PCR efficiency using primer set 582f/728r ranged from 76% to 82% (corresponding to slope of -3.96 ± 0.12), comparable to the range reported by Ritalahti et al. (2006) (slope of -3.80 ± 0.22) for using primer set Dhc1200F/Dhc1271R in conjunction with plasmid DNA containing “Dehalococcoides” strain BAV1 16S rRNA gene as template. The y-axis of the calibration curves indicates the number of PCR amplification cycles needed to detect a certain number of gene copies. A higher y-intercept corresponds to a lower detection limit. For example, in this case, it would only take 28 cycles to detect 1000 gene copies/reaction for strain BL-DC-9, but would take 34 cycles to detect the same number of “Dehalococcoides” 16S rRNA gene copies/reaction using the methods employed here.

5.3.5 Application of qPCR in Environmental Samples

qPCR was applied to DNA extracts from groundwater samples to quantify concentrations of the bacterial group represented by strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” strains in the DNAPL source zone of the PPI Superfund site. The pH measured from each sampling wells were listed in Table 5.5. In a preliminary test conducted using the original non-diluted DNA extracts, no increase in fluorescence beyond background was observed in any of the qPCR reactions, indicating the presence of PCR inhibiting compounds (data not shown). When DNA extracts were diluted 10 \times or 100 \times to minimize the concentrations of inhibitory compounds, qPCR was successful at both dilution levels. More measurements, however, dropped below the quantification limit at the 10 $^{-2}$ dilution level. Thus, qPCR reactions were performed at the 10 $^{-1}$ dilution level followed by melting curve analysis to check for presence of non-specific amplification products. PCR amplicons generated using primer sets BL-DC-631f/ BL-DC-796r or 528f/728r all produced one unique melting curve peak, indicating primer specificity in analysis of these environmental samples (data not shown).

Table 5.5. pH of the groundwater collected from the PPI superfund site

Well ID no.	Groundwater pH
W-0627-2	4.860
W-0721-1	6.644
W-0726-4	5.491
W-0820-1	6.937
W-0822-3	5.916
W-0823-2	5.824
W-0825-1	6.329
W-0828-1	4.238

Quantification of 16S rRNA genes was performed in triplicate for each primer combination and each diluted DNA extract. Results are presented in Fig. 5.7. The 16S rRNA genes of the bacterial group represented by strains BL-DC-8 and BL-DC-9 were detected in all eight groundwater samples with concentrations ranging from $1.33 \pm 0.09 \times 10^2$ to $1.88 \pm 0.07 \times 10^6$ copies/mL groundwater. “Dehalococcoides” 16S rRNA genes were detected in six groundwater samples (wells W-0721-1, W-0726-4, W-0823-2, W-0828-1, W-0820-1, and W-0825-1). The concentrations were sufficiently low (i.e., below the linear range of the calibration curve), however, that the “Dehalococcoides” 16S rRNA gene copy numbers could be quantified only in DNA extracts originating from two of the wells, W-0820-1 and W-0823-2. The samples from well W-0627-2 and W-0822-3 had no PCR amplification after 40 cycles. For samples in which “Dehalococcoides” amplicons were detected, concentrations ranged from $<2.8 \times 10^3$ copies/mL (the lower limit of the linear range of the calibration curve) to $5.84 \pm 0.20 \times 10^5$ /mL. Highest concentrations observed for strains BL-DC-8/BL-DC-9 and “Dehalococcoides” were in the sample from well W-0823-2, with concentrations of $1.88 \pm 0.07 \times 10^6$ copies/mL and $5.84 \pm 0.20 \times 10^5$ copies /mL, respectively. PCR amplicons generated using primer sets BL-DC-631f/

BL-DC-796r and 528f/728r all produced a single melting curve peak, indicating primer specificity in analysis of these environmental samples (data not shown).

There are three issues that may be viewed as potential discrepancies between results from qPCR analyses and results from the studies employing direct or nested PCR. First, Universal bacterial primers amplified DNA in the extract of well W-0828-1 in the qPCR approach but not in the nested-PCR approach. This may have resulted from use of a different primer universal bacterial primer set (Bac1055YF/Bac1392R versus 530f/900r) which may have higher amplification efficiency due to smaller amplicon size, PCR reagent composition, and the use of more thermal cycles (40 versus 35) used in the qPCR reactions. Second, DNA amplification using the “Dehalococcoides”-specific primer set 582f/728r resulted in amplification of DNA extracts from well W-0822-3 in the nested PCR approach but not using qPCR. This may have resulted from a larger total number of PCR amplification cycles (35 using universal bacterial primers plus 35 using “Dehalococcoides”-specific primers in the nested PCR approach versus 40 cycles in the qPCR protocol). Third, DNA amplification using the BL-DC-631f/ BL-DC-796r primer set resulted in amplification of DNA extracts from well W-0828 in qPCR but not in the nested PCR approach. This latter apparent discrepancy cannot be readily explained but may have resulted from differences in PCR reagent composition.

Collectively, results from qPCR experiments further confirmed results from nested PCR experiments, that bacteria sharing high 16S rRNA gene sequence identity with strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” populations were widely distributed in the DNAPL source zone of the PPI site. Markedly higher 16S rRNA gene copy numbers of BL-DC-strains ($1.88 \pm 0.07 \times 10^6/\text{mL}$) and “Dehalococcoides” strains ($5.84 \pm 0.20 \times 10^5/\text{mL}$) were detected in

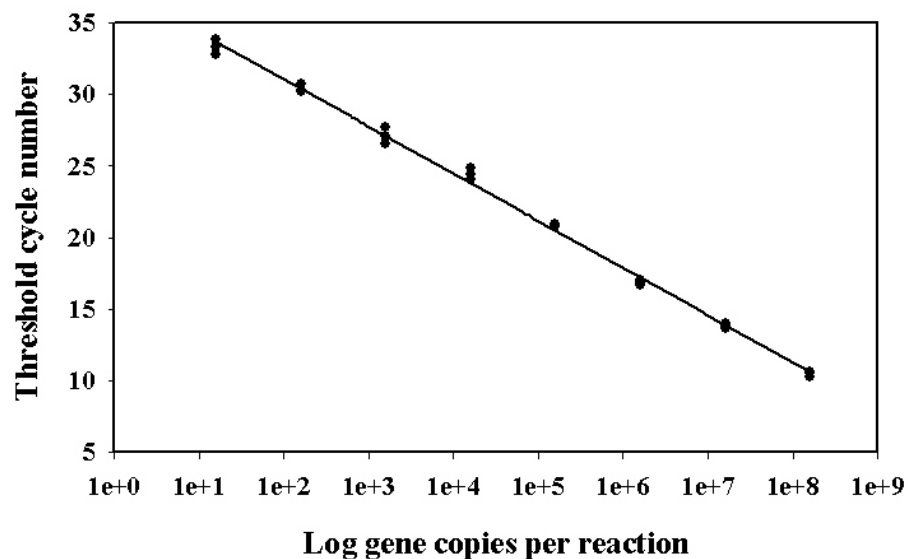


Figure 5.5. Calibration curve for strain BL-DC-8/BL-DC-9 16S rRNA gene copy numbers prepared using primers BL-DC-631f/BL-DC-796r in conjunction with serial dilution of plasmid DNA. (●)16S rRNA gene.

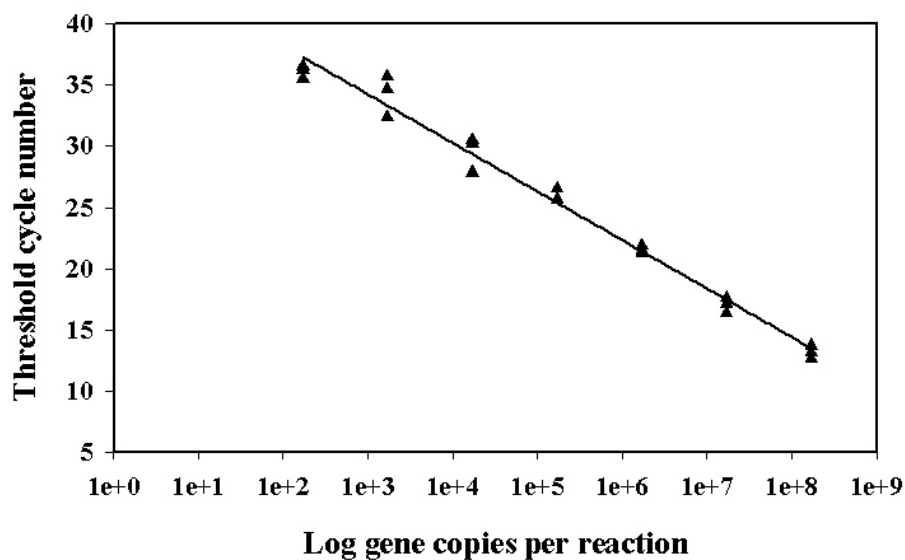


Figure 5.6. Calibration curve for "Dehalococcoides" strains 16S rRNA gene copy numbers prepared using primers 582f/728r in conjunction with serial dilution of plasmid DNA. (▲)16S rRNA gene.

groundwater sampled from well W-0823-2. These concentrations were 3 to 4 orders of magnitude higher than concentrations determined for the other wells sampled.

Total bacterial 16S rRNA gene copy numbers in the eight groundwater samples ranged from $8.40 \pm 1.25 \times 10^3$ to $2.38 \pm 0.86 \times 10^7$ copies/mL groundwater. This is consistent with the observation of 3×10^7 cells /mL via direct microscopic counts using groundwater collected from the nearby well W-1024-1, also located in the DNAPL source zone of the PPI site (Bowman *et al.*, 2006).

5.3.6 Application of qPCR in Dehalogenating Mixed Culture

qPCR was employed to quantitatively investigate growth of the bacterial group represented by strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” strains in a mixed culture that dehalogenated 1,1,2-TCA to ethene. Panel A in Fig. 5.8 presents the concentrations of 1,1,2-TCA and its dechlorination products observed over time. Error bars represent standard deviation of triplicate bottles sacrificed at each time step. As shown in the figure, vinyl chloride transiently accumulated as a dechlorination product, reaching a maximum concentration of 2.56 ± 0.54 $\mu\text{mol/bottle}$ (corresponding to 11.5% of the total 1,1,2-TCA supplied) during the 49 day incubation period. The vinyl chloride, however, was subsequently consumed, and by the end of the incubation period there was essentially complete stoichiometric conversion of 1,1,2-TCA to the non-chlorinated final product ethene. A linear fit to experimentally determined ethene concentrations versus time in days using a simple linear regression model resulted in a calculated zero-order ethene production rate of 12.27 ± 0.35 $\mu\text{mol}/(\text{L}\cdot\text{day})$ (where the volume term in the denominator of the units specified refers to the liquid volume in the bottle).

At the beginning of the experiment, 10% H_2 was supplied in the gas headspace, which is roughly three times the stoichiometric requirement for complete dehalogenation of 1,1,2-TCA to

Table 5.6. Amplification efficiencies and quantification linear ranges of 16S rRNA gene calibration curves

Target gene	Template	Amplification efficiency ^a	Slope ^b	y-intercept ^b	R2	Linear range (copies/25 µL reaction)	Detection limit (copies/ 25 µL reaction)
Bacterial 16S rRNA	BL-DC-9 plasmid	94%-102%	-3.37±0.10	41.86±0.59	0.983	2.24×10 ² -2.24×10 ⁸	2-224
Dehalogenimonas 16S rRNA	BL-DC-9 plasmid	100%-102%	-3.30±0.03	36.34±0.17	0.998	2.24×10 ¹ -2.24×10 ⁸	2-22
“Dehalococcoides” 16S rRNA	DHC-4 plasmid	76%-82%	-3.96±0.12	46.09±0.64	0.984	1.70×10 ² -1.70×10 ⁸	17-170

^a Amplification efficiency was calculated according to equation: $\eta = 10^{\left(-1/\text{slope}\right)} - 1$

^b Mean values and standard deviations were calculated using simple linear regression model by SAS.

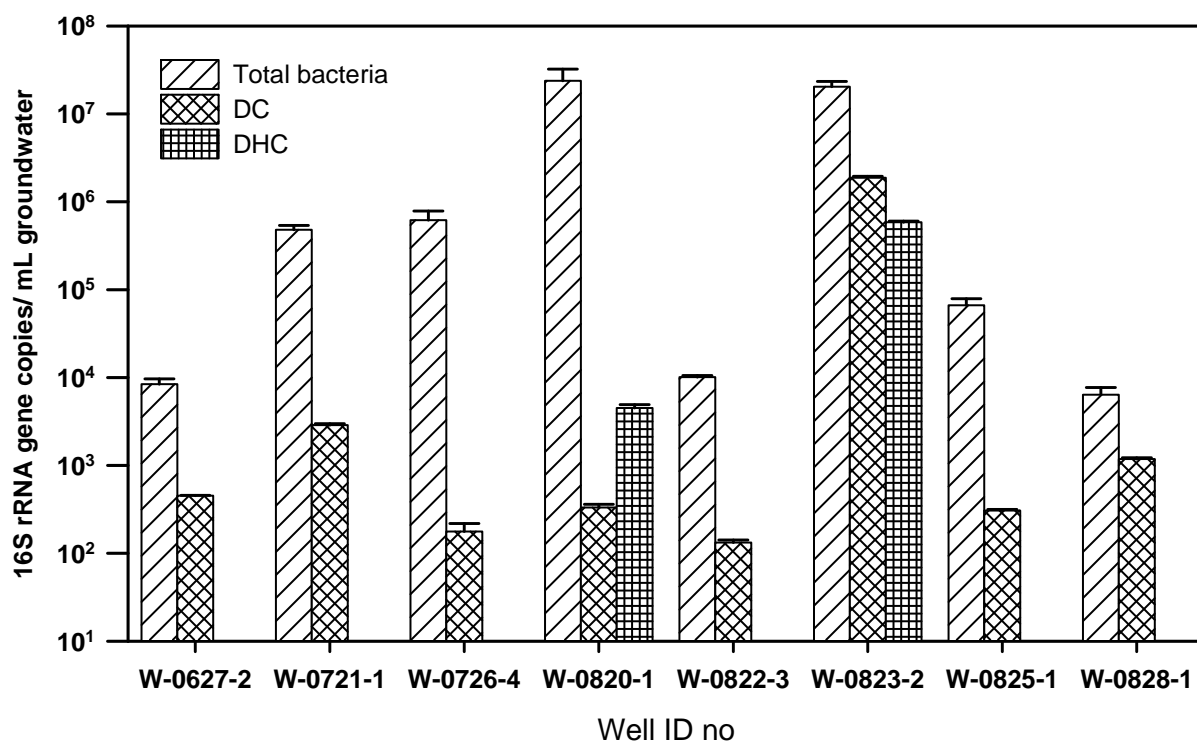


Figure 5.7. qPCR quantification of total bacteria, BL-DC- (DC), and “Dehalococcoides” (DHC) 16S rRNA gene copies in groundwater from the DNAPL source zone at the PPI Superfund site. Samples with gene copy concentrations below quantification or detection limits are not plotted in this figure. The total bacterial 16S rRNA gene copy numbers were calculated with the *E. coli* calibration curve.

ethene (see calculations in Appendix B). However, H₂ was found to be depleted when analyzed on day 31, at which time only 56% of the 1,1,2-TCA was dehalogenated into ethene, and the dehalogenation rates appeared to temporarily decrease or stop. After spiking the bottles with H₂ to reach a concentration of 5% (v/v) in the headspace on day 31, dehalogenation resumed and the remaining 1,1,2-TCA and vinyl chloride was dehalogenated by day 49.

The numbers of BL-DC-8 and BL-DC-9 and “Dehalococcoides” strains 16S rRNA gene copies during 1,1,2-TCA dehalogenation as quantified by qPCR are presented in Fig. 5.8 panel B. Error bars represent the standard deviation from triplicate bottles sacrificed at that sampling time point. As shown in the figure, there was an approximately 7-day long initial lag period during which gene copy numbers were relatively stable, corresponding to the time interval during which 1,1,2-TCA remained near its initial concentration. Thereafter, “Dehalococcoides” strains were found to grow simultaneously with the BL-DC- bacterial group. “Dehalococcoides” strains are the only bacterial group known to dehalogenate vinyl chloride, and in this case, they may have been supplied with vinyl chloride from dehalogenation of 1,1,2-TCA by bacteria similar or identical to strains BL-DC-8 and BL-DC-9. The results suggest that complete 1,1,2-TCA dechlorination to ethene may involve multiple dehalogenating populations.

In pure culture, strains BL-DC-8 and BL-DC-9 dehalogenated the same amount of 0.5 mM 1,1,2-TCA within three weeks or less (see Fig. 3.5). The starting concentration of cells (measured in terms of 16S rRNA gene copies) was much higher in the pure culture experiments.

The net yield of 16S rRNA genes of “Dehalococcoides” strains was calculated by plotting the increase in ethene quantity versus increase in “Dehalococcoides” gene copies for each time step. This calculation explicitly assumed that “Dehalococcoides” strains were responsible for all ethene production using vinyl chloride as the electron acceptor and that “Dehalococcoides” strains did not utilize other electron acceptors (i.e., 1,1,2-TCA). The net yield of the genus

represented by strains BL-DC-8 and BL-DC-9 was similarly calculated by plotting the disappearance of 1,1,2-TCA versus increase in gene copies for each time step. This calculation explicitly assumed that the phylotype represented by strains BL-DC-8 and BL-DC-9 was responsible for transforming all 1,1,2-TCA consumed, with vinyl chloride as the sole product, and that other electron acceptors were not utilized. Graphs are presented in Appendix B. The net yield of 16S rRNA gene of “Dehalococcoides” with vinyl chloride was calculated to be $6.78 \pm 0.64 \times 10^7$ [16S rRNA gene copies/ $\mu\text{mol Cl}^-$ released] ($R^2=0.7248$), close to that of previously reported for “Dehalococcoides” strains (He *et al.*, 2003b, 2005, also see Table 3.1). The net yield of the BL-DC-8/BL-DC-9 phylotype based on assumptions listed above, however, was calculated to be $2.01 \pm 0.32 \times 10^5$ [16S rRNA gene copies/ $\mu\text{mol Cl}^-$ released] ($R^2=0.4862$), which is approximately two orders of magnitude lower than what was observed for strains BL-DC-8 and BL-DC-9 when grown with 1,1,2-TCA in pure culture (see Table 3.1 and Section 3.3.2). It is not clear from the data collected whether this apparent discrepancy reflects the fact that the net yield of the BL-DC-8/BL-DC-9 phylotype was lower in the mixed culture than in pure culture or whether it reflects the fact dechlorinating microorganisms other than the BL-DC-8/BL-DC-9 phylotype were responsible for transforming a portion of the 1,1,2-TCA consumed.

5.4 Discussion

Three “Dehalococcoides” specific primer sets reported by Hendrickson *et al.* (2002) were demonstrated to amplify the corresponding 16S rRNA gene regions of strains BL-DC-8 and BL-DC-9. It appears that this non-specific amplification was influenced more by the location rather than the total number of base mismatches. For example, primer sets Fp DHC 1/ Rp DHC 692 and Fp DHC 587/ Rp DHC 1090 both have a combined total of five base mismatches from the sequence of strain BL-DC-9. The mismatched bases, however, are not located at the priming ends. This allowed non-specific amplification of BL-DC-9 DNA when the relatively low

annealing temperature of 55 °C was employed in PCR reactions. In contrast, although primer set 1f/259r has only two base mismatches from the sequence of strain BL-DC-9, no amplification was observed when annealed at 59 °C. This likely resulted because the two mismatched bases are located at the priming ends. Non-specific amplification (which could lead to the incorrect conclusion that “Dehalococcoides” were present in a sample when in fact they are not) could be avoided by simply elevating the annealing temperature. Such an approach, however, will likely result in lower PCR amplification efficiency and therefore detection sensitivity. Consequently, the primer combinations of Fp DHC 1/ Rp DHC 692, Fp DHC 385/ Rp-DHC 806 and Fp DHC 587/ Rp DHC 1090 are not ideal choices for detection of “Dehalococcoides” sp. in mixed cultures or environmental samples. Additionally, among the previously reported “Dehalococcoides”-specific primers, Fp DHC 1, Fp DHC 774, Fp DHC 385, and Rp DHC 806 contain zero or one base mismatches from the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9. Consequently, they should be used cautiously in combination with a highly specific forward or reverse primer or avoided altogether when attempting to uniquely amplify DNA from “Dehalococcoides” strains.

By employing specific primers in the groundwater samples, the widespread distribution of bacteria similar or identical to BL-DC-8 and BL-DC-9 in the DNAPL source zone was determined at the Brooklawn portion of the PPI Superfund site. Until relatively recently, microbial reductive dehalogenation in DNAPL source zones was generally considered negligible due to the assumed toxicity of high concentrations of chlorinated solvents (Yang and McCarty, 2002). Recent studies, however, have demonstrated that organisms, including “Dehalococcoides” strains, could survive and actively dehalogenate chlorinated ethenes in the presence of saturation concentrations of chlorosolvents. Furthermore, it was demonstrated that this can biologically

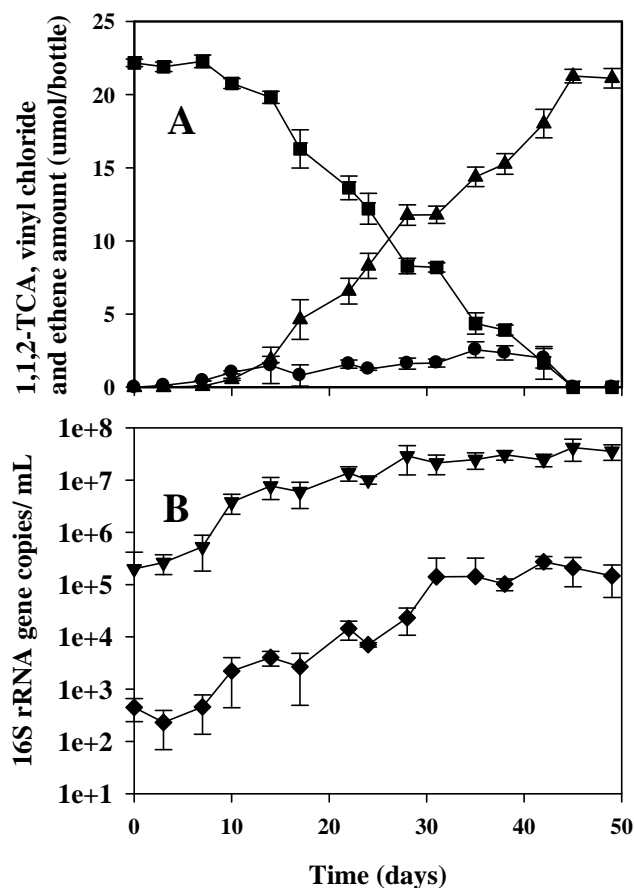


Figure 5.8. Dehalogenation of 1,1,2-TCA in enrichment culture microcosm. (■) 1,1,2-TCA; (▲) ethene; (●) vinyl chloride; (▼) “Dehalococcoides” 16S rRNA gene; (◆) strains BL-DC-8, BL-DC-9 16S rRNA gene.

stimulate DNAPL dissolution at rates appreciably faster than in abiotic systems (Yang and McCarty, 2000, 2002; Dennis *et al.*, 2003; Sleep *et al.*, 2006).

The widespread detection of dehalogenating bacteria in the DNAPL source zone is consistent with the previous report that dehalogenation daughter products (e.g., vinyl chloride, ethene) and high concentrations of bacteria ($>3 \times 10^7$ cells/mL groundwater) were found in the DNAPL source zone at this site (Bowman *et al.*, 2006).

The highest concentrations of the phylotype represented by strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” species were observed in the sample from well W-0823-2, with

concentrations of $1.88 \pm 0.07 \times 10^6$ /mL and $5.84 \pm 0.20 \times 10^5$ /mL groundwater, respectively. These concentrations are orders of magnitude higher than concentrations determined for the other wells sampled, and it may indicate the occurrence of a particularly rapid natural attenuation process at this location.

The gene copy numbers determined using primers BL-DC-631f/BL-DC-796r ($1.18 \pm 0.04 \times 10^3$ copies/mL, representing bacteria similar or identical to BL-DC-8 and BL-DC-9) were 18.6% of the total bacterial 16S rRNA gene copies ($6.37 \pm 1.34 \times 10^3$ copies/mL, determined using universal bacterial primers Bac1055YF/Bac1392R) for the groundwater sampled from well W-0828-1. Such a high percentage of dehalogenating bacteria in the microbial community has only been reported previously for enrichment cultures where a readily available supply of electron donors (e.g., H₂) and other favorable growth conditions are provided (Duhamel *et al.*, 2004; Bowman *et al.*, 2006; He *et al.*, 2007).

In the 1,1,2-TCA enrichment culture, the concentration of the bacterial group represented by strains BL-DC-8 and BL-DC-9 was found to be approximately two orders of magnitude lower than “Dehalococcoides” strains. This is a much lower relative abundance than what was observed for groundwater samples tested in this study. Because comprehensive studies of the influence of various growth conditions (e.g., pH, temperature, chlorinated solvent concentrations) on the relative growth rates of various dehalogenating bacteria have not yet been reported, the reasons for this observation are not yet clear. It is possible that the bacterial group represented by strains BL-DC-8 and BL-DC-9 grows at a higher relative rate and are better able to survive in moderately acidic (groundwater pH was approximately 5, versus pH of 7 in the enrichment culture) or higher solvent concentration environments (groundwater chlorinated solvent concentrations were approximately an order of magnitude higher than in the enrichment culture).

It is also possible that other as yet unknown species present in the 1,1,2-TCA enrichment culture could have dehalogenated 1,1,2-TCA to vinyl chloride.

5.5 Conclusions

The specificity of twelve combinations of previously published primers specific to “Dehalococcoides” strains 16S rRNA gene was re-evaluated in this study. Three of the primer sets, Fp DHC 1F/ Rp DHC 692r, Fp DHC 385/ Rp 806 and Fp DHC 587/ Rp DHC 1090r, were demonstrated to cause non-specific amplification of a bacterial group other than “Dehalococcoides” strains (i.e., strains BL-DC-8 and BL-DC-9) when employing PCR thermal protocols described previously in the literature. Non-specific amplification could be avoided by increasing the annealing temperature. Additionally, among the previously reported “Dehalococcoides”-specific primers, Fp DHC 1, Fp DHC 774, Fp DHC 385, and Rp DHC 806 contain zero or one base mismatches from the 16S rRNA gene sequences of strains BL-DC-8 and BL-DC-9. Consequently, they should be used cautiously in combination with a highly specific forward or reverse primer or avoided altogether when attempting to uniquely amplify DNA from “Dehalococcoides” strains.

Based on the 16S rRNA gene sequences derived from strains BL-DC-8 and BL-DC-9, oligonucleotide primers and PCR-based protocols were developed. These protocols were successfully applied to investigate the spatial distribution and relative abundance in chlorinated compounds contaminated groundwater at the PPI site. The bacterial group represented by strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” strains were found to be widely distributed in the DNAPL source zone at the PPI Superfund site. qPCR results revealed that at some locations the phylotype represented by strains BL-DC-8 and BL-DC-9 was present at a relatively high concentration relative to the total bacterial community. This suggests that they may play an important functional role in dehalogenation even in the presence of high

concentrations of chlorosolvents found at the site. Bacterial concentrations measured over time in a 1,1,2-TCA dechlorinating mixed culture using qPCR demonstrated that the phylotype represented by strains BL-DC-8 and BL-DC-9 can grow concurrently with “Dehalococcoides” strains, and it suggests that multiple bacterial groups can contribute to complete reductive dehalogenation of chlorinated in mixed cultures.

The 16S rRNA gene specific primers, PCR and qPCR protocols developed in this study may be useful to track the concentrations of the bacterial group represented by strains BL-DC-8 and BL-DC-9 in environments, including bioaugmented sites.

CHAPTER 6. OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Overall Conclusions

An anaerobic enrichment culture able to reductively dehalogenate 1,1,2-TCA was established using chlorinated compounds contaminated groundwater collected from the PPI Superfund site as the inoculum. This confirmed previous findings that microorganisms with the ability to anaerobically biotransform chlorinated solvents are present within the DNAPL source zone in the Brooklawn portion of the PPI site.

Using a dilution-to-extinction procedure, several bacteria with high similarity (>98%) to “Dehalococcoides” strains in 16S rRNA gene sequences were isolated from the 1,1,2-TCA degrading enrichment culture. Two additional strictly anaerobic strains, designated as BL-DC-8 and BL-DC-9, that are somewhat related to “Dehalococcoides” strains (90% 16S rRNA gene sequence identity) were also isolated. Because they are quite distantly related to previously characterized bacteria, strains BL-DC-8 and BL-DC-9 were characterized using a polyphasic approach. Phylogenetic analyses based on 16S rRNA gene sequences placed both strains within a new lineage within the *Chloroflexi*. Their closest previously cultured relatives are the “Dehalococcoides” strains, but with only 90% sequence identity, indicating that they represent a new genus.

Strains BL-DC-8 and BL-DC-9 were found to grow best at neutral pH and in the temperature range of 28 °C to 34 °C. Both strains were strict anaerobes but could tolerate short-term oxygen exposure. Strains BL-DC-8 and BL-DC-9 were shown to dehalogenate a variety of chlorinated alkanes including 1,1,2,2-TeCA, 1,1,2-TCA, 1,2-DCA, 1,2,3-TCP and 1,2-DCP in growth related processes. Growth was observed only when a vicinally chlorinated alkane was supplied as an electron acceptor and hydrogen was supplied as an electron donor. All observed

dehalogenation reactions by the two strains appear to involve dihaloelimination.

It was demonstrated for the first time that 1,2,3-TCP could be reductively dehalogenated in a growth-linked process by strains BL-DC-8, BL-DC-9. Both biotic and abiotic processes were found to be involved in the pathway for complete dehalogenation of 1,2,3-TCP. Collectively, these findings suggest that anaerobic biological methods may be suitable for remediation of environmental 1,2,3-TCP contamination. Metabolites and DNA sequences identified during the course of this study may prove useful as biomarkers to track the biodegradation of 1,2,3-TCP.

Based on the 16S rRNA gene sequences derived from strains BL-DC-8, BL-DC-9, specific oligonucleotide primers and PCR-based protocols were developed. These were successfully applied to investigate their spatial distribution and relative abundance in chlorosolvent-contaminated groundwater at the PPI site. The bacterial group represented by strains BL-DC-8 and BL-DC-9 as well as “Dehalococcoides” strains were found to be widely distributed in the DNAPL source zone at the PPI Superfund site. qPCR results revealed that some locations the BL-DC-8 and BL-DC-9 phylotype were present at a relatively high concentrations relative to the total bacterial community. This suggests that they may play an important functional role in dehalogenation even in the presence of high concentrations of chlorosolvents found at the site. Bacterial concentrations measured over time in a 1,1,2-TCA dechlorinating mixed culture using qPCR demonstrated that the phylotype represented by strains BL-DC-8 and BL-DC-9 can grow concurrently with “Dehalococcoides” strains, and it suggests that multiple bacterial groups can contribute to complete reductive dehalogenation of chlorinated in mixed cultures.

Overall, the discovery and characterization of the novel isolates BL-DC-8 and BL-DC-9 has expanded the body of knowledge related to the diversity and potential functions of

halorespiring microbial populations, particularly for the bacteria able to transform chlorinated alkanes. The information derived from this study may be prove useful in improving decision making related to strategies for clean up sites contaminated with chlorinated alkanes.

6.2 Recommendations for Future Research

Additional studies are required to further investigate the dehalogenation activities of bacteria similar or identical to BL-DC-8 and BL-DC-9 in real environments and under conditions in which multiple chlorinated compounds are present in free-phase or near saturated concentrations.

In addition to investigating the geographic distribution of stains BL-DC-8 and BL-DC-9 at contaminated sites, it is recommend that oligonucleotide primers and PCR-based protocols developed in this study also be applied to track in-situ bioremediation performance and the transport patterns of strains BL-DC-8, BL-DC-9 in the simulated porous media. This would allow further assessment of their suitability for use in bioaugmentation of environments in which contaminant transformation is limited by the lack of a microbial community able to biotransform chlorinated alkanes.

In recent years, several dehalogenase enzymes which catalyze the dehalogenation of PCE, TCE and vinyl chloride have been identified and the corresponding DNA sequences coding for their production have been determined (Magnuson *et al.*, 2000; Krajmalnik-Brown *et al.*, 2004; Muller *et al.*, 2004; Nijenhuis and Zinder, 2005; Adrian *et al.*, 2007). It is recommended that future studies be conducted to characterize the chloroalkanes dehalogenase(s) contained by strains BL-DC-8 and BL-DC-9. It is anticipated that this would facilitate a better understanding of the biochemical pathways leading to the unique metabolic pathways that these strains posses. It would also likely aid in development of unique DNA sequences useful for assessing and tracking capacity microbial populations with the ability to degrade 1,2,3-TCP. Genomic DNA

from strain BL-DC-9 has been submitted to the US Department of Energy (DOE) Joint Genome Institute (JGI) Community Sequencing Program (CSP) for full genome sequencing as a step toward achieving the goals of this suggested future research (Appendix C).

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APPENDIX A: MELTING TEMPERATURE CALCULATION

The following formula, recommended by Operon Biotechnologies, Inc. (Huntsville, AL) for the calculation of primers ranging from 20 to 100 residues and sodium concentrations ranging from 0.01 M to 1.0 M, was used to calculate melting temperatures reported in this dissertation.

$$T_m = 81.5 + 16.6 \times \log [Na^+] + 41 \times (G+C) / \text{length} - 500 / \text{length}$$

Where: $[Na^+]$ = sodium concentration [M]

(G+C) = number of nucleotides comprised of either G or C

Length = total number of nucleotide residuals in the primer

Values reported in this dissertation were for an assumed $[Na^+]$ concentration of 0.1 M.

APPENDIX B: GROWTH YIELD CALCULATION

The net yield of “Dehalococcoides” sp. was calculated by plotting the increase in ethene quantity versus increase in “Dehalococcoides” gene copies for each time step (Fig. C.1 A). The net yield of the phylotype represented by strains BL-DC-8 and BL-DC-9 was similarly calculated by plotting the disappearance of 1,1,2-TCA (expressed as the total amount of vinyl chloride plus ethene) versus increase in gene copies for each time step (Fig. C.1 B).

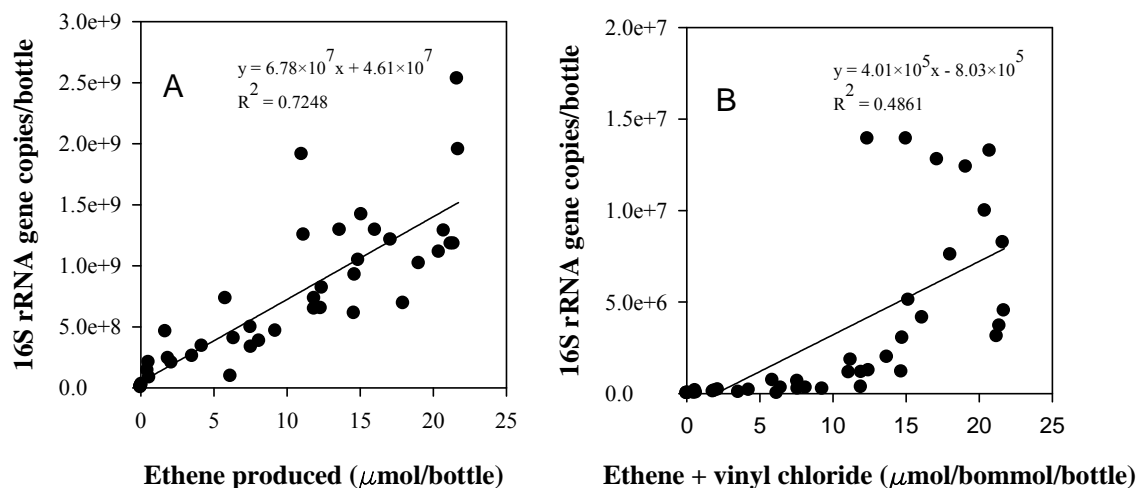


Figure C.1. Growth yield of “Dehalococcoides” sp. (A) and the bacterial group represented by strains BL-DC-8 and BL-DC-9 (B) in the 1,1,2-TCA dechlorinating enrichment culture.

APPENDIX C: BACTERIAL GENOMIC DNA PREPARATION PROTOCOLS

This appendix presents the methods used to prepare genomic DNA from strain BL-DC-9 for sequencing via the US Department of Energy (DOE) Joint Genome Initiative (JGI) Community Sequencing Program (CSP). To minimize DNA shear and improve DNA quality, a CTAB DNA extraction protocol was used in here as recommended by JGI to prepare the DNA sample for genome sequencing.

Materials and Methods

Chemicals

Hexadecyltrimethyl ammonium bromide (CTAB, Sigma H-6269); TE buffer (10mM tris; 1 mM EDTA, pH 8.0, Ambion 9858); Lysozyme (100 mg/mL, Sigma L-6876); Proteinase K (10 mg/mL, Qiagen 19131); SDS (Sigma L-4522); Chloroform (Sigma C-2432); IsoAmyl alcohol (Sigma I-9392); Phenol (Sigma P-4557); Isopropanol (VWR PX-1835-14); RNase A (100mg/mL, Qiagen 19101); DNA size markers and concentration standards were provided by DOE Joint Genome Institute.

CTAB/NaCl Solution

Dissolve 4.1 g NaCl in 80 ml of water and slowly add 10 g CTAB while heating at 65°C and stirring for 30 to 40 min to completely dissolve CTAB. Adjust the final volume to 100 ml and sterilize by filter or autoclave.

Biomass Collection

2 L of 1,1,2-TCA grown culture was centrifuged in 30 mL polypropylene centrifuge tubes at 10,000×g for 10 min at 4 °C. The supernatant was discarded and the precipitated pellet was resuspended with 2.4 mL 1×TE buffer. The total volume was adjusted with 1×TE buffer to lower OD⁶⁰⁰ to 1.0 or less and then cell solution was evenly distributed into 4 2.0 mL eppendorf tubes.

Genomic DNA Extraction

Add 20 μ L lysozyme to each tube and incubate at room temperature for 5 min. Then add 8 μ L Proteinase K and 40 μ L 10% SDS into each tube and incubate at 37°C for 1 hr. After the incubation, add 100 μ L 5M NaCl and 100 μ L CTAB/NaCl solution (pre-heated to 65°C) and incubate at 65°C for 10 min. Add 0.5 mL chloroform: isoamyl alcohol (24:1) to each tube and centrifuge at 13,000 rpm for 10 min. Transfer the upper aqueous phase without touching any debris to a new eppendorf tube. Add 0.5 mL phenol: chloroform: isoamyl alcohol (25:24:1) to each tube and centrifuge again at 13,000 rpm for 10 min. Transfer the upper aqueous phase and add 0.6 \times volume isopropanol (-20°C) and incubate at room temperature for 30 min. Centrifuge the tubes at 13,000 rpm for 15 min and carefully remove all the supernatant. Add 1 mL 70% alcohol to each tube and centrifuge at 13,000 rpm for 5 min. Carefully discard the supernatant and dry the DNA precipitation at room temperature for 30 min. Resuspend DNA in 25 μ L 1 \times TE buffer contains 0.1 mg/mL RNase A. Let the DNA dissolve in TE buffer overnight and combine 4 tubes in one.

DNA Quantification

0.5~1 μ L genomic DNA sample was loaded and visualized in 1% 1 \times TAE agarose gel with DNA standard and DNA marker to determine the quality and quantity. 9V/cm voltage was applied to the gel for 90 min to move the genomic DNA \geq 2 cm. DNA concentration was determined by using gel imaging software Quantity One (BioRad Laboratories, Hercules, CA)

Results

Bulk DNA molecular weight meets the requirement of greater than 23 kb by comparing with DNA marker 2 (Fig. D.1). The DNA quantity calibration curve was made by plotting DNA standards (31, 63, 125, 250, 500 ng) against their corresponding band volume (intensity \times band area). The DNA concentrations for sample I, II and III were found to be 411.28, 308.98 and

246.94 ng/ μ l, respectively. The DNA concentration for each sample (>100 ng/ μ l) and the total combined DNA quantity of three samples is 96.72 μ g, which meet the JGI's requirements.

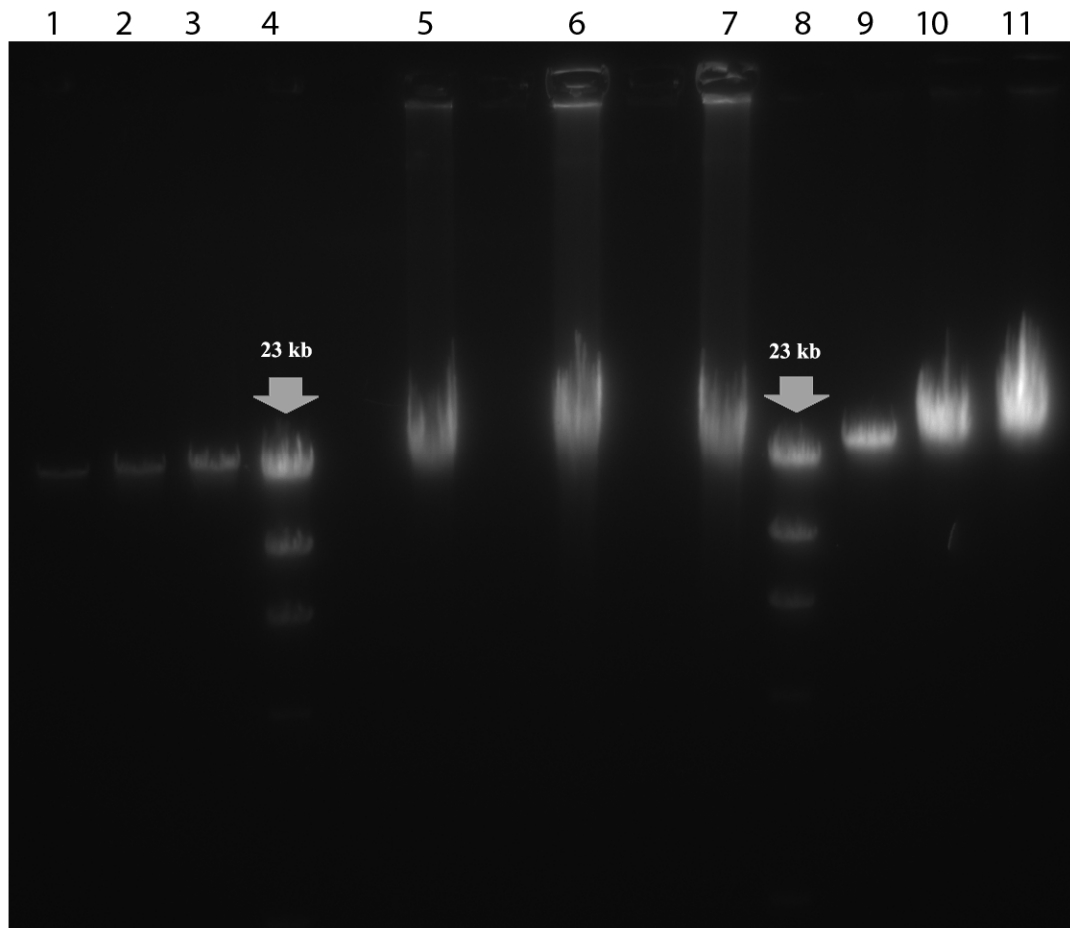


Figure D.1. Gel image (unsaturated pixels) of strain BL-DC-9 genomic DNA. Lane 1, 15 ng standard (5 μ L); lane 2, 31 ng standard (5 μ L); lane 3, 63 ng standard (5 μ L); lane 4, DNA marker 2 (5 μ L); lane 5, strain BL-DC-9 genomic DNA extract (0.5 μ L) batch I from 2 L culture (7/26/08); lane 6, strain BL-DC-9 genomic DNA extract (1 μ L) batch II from 2 L culture (9/15/08); lane 7, strain BL-DC-9 genomic DNA extract (1 μ L) batch III from 2 L culture (9/16/08); lane 8, DNA marker 2 (5 μ L); lane 9, 125 ng standard (5 μ L); lane 10, 250 ng standard (5 μ L); lane 11, 500 ng standard (5 μ L).

VITA

Jun Yan was born in Nanjing, Jiangsu, China, in November, 1978. Jun Yan received his Bachelor of Science in Environmental Sciences and Engineering degree from Nanjing University, China, in May 2000. Afterwards, he continued his graduate studies in Nanjing University and obtained his Master of Science in Environmental Sciences degree in May 2003. In August 2003, Jun Yan enrolled in the doctoral program in the Department of Civil and Environmental Engineering at the Louisiana State University and Agriculture and Mechanical College under the direction of Dr. William M. Moe, to study reductive dehalogenation of chlorinated compounds. Currently, Jun Yan is a candidate for the Doctor of Philosophy degree in civil engineering, and expected to be awarded the degree in May 2009.